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Isolation of microorganisms which utilize acidic D-amino acid oligomers

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Abstract

The D-amino acid octamers (D-Glu)$_8$ and (D-Asp)$_8$ were synthesized in order to screen for new microbial degraders of unnatural D-amino acid peptides. We have successfully isolated the microorganisms from soil acclimated to a medium containing the oligopeptides; they were classified as *Klebsiella ornithinolytica, Delftia acidovorans*, and α-Proteobacteria.
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Keywords: D-Aspartic acid octamer; D-Glutamic acid octamer; *Klebsiella ornithinolytica*; *Delftia acidovorans*; α-Proteobacteria

1. Introduction

We are interested in exploiting unknown catalytic abilities of microorganisms. The development of enzymes catalyzing new reactions will contribute not only to the basic enzymology, but also to a variety of synthetic and industrial applications [1]. We have been successful in characterizing several new D-stereospecific peptidases [2]. We discovered a new enzyme D-aminopeptidase (DAP) from *Ochrobactrum anthropi*, which was isolated from soil with a medium containing D-alanine amide as a sole source of nitrogen [3]. Its structure, function and application to D-stereospecific hydrolysis of racemic amino acid amides and formation of peptide bonds by an aminolysis reaction have been described [4–7]. We isolated a degrader of a synthetic peptide (D-Phe)$_4$ and characterized a new enzyme “alkaline D-peptidase (ADP)” responsible for the degradation from *Bacillus cereus* [8]. ADP is applicable to the synthesis of aromatic D-amino acid-containing peptides [9]. We recently characterized aromatic D-amino acid amidase, and cloned and sequenced the gene for this enzyme from *Ochrobactrum anthropi* SV3 [10,11]. This enzyme is expected to be useful in the D-stereospecific resolution of aromatic amino acid amides. From their primary structures and other biochemical evidence, we discovered that all these new D-stereospecific peptidases belong to the “penicillin-recognizing enzymes”, which include β-lactamase and penicillin binding proteins [12–14].

We speculated that the microbial degrader of acidic D-amino acid peptides would possess an enzyme system to utilize these compounds. Microbial and

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biochemical characterization of these enzymes will provide knowledge for understanding their chemistry, catalytic mechanisms, and evolution.

We recently synthesized (D-Asp)$_8$ and (D-Glu)$_8$ having amide bonds between the 1-carboxylic acid and the 2-amino group. (D-Asp)$_8$ and (D-Glu)$_8$ are new compounds as far as we can determine from the literature. Microorganisms capable of assimilating (D-Asp)$_8$ and (D-Glu)$_8$ as a sole carbon and nitrogen source were screened from soil samples.

2. Materials and methods

2.1. Materials

^{1}H NMR spectra were recorded using a JEOL JNM-LA400 spectrophotometer. D-Asp, D-Glu, benzyl alcohol, sulfuric acid, citric acid and triethylamine (TEA) were purchased from Wako Pure Chemicals (Japan). Di-*tert*-butyloxycalbonyl anhydride ((BOC)$_2$ O), *N*-hydroxysuccinimide alcohol (HONSu), dicyclohexylcarbodiimide (DCC) and 4N HCl in dioxane were purchased from Kokusan Chemicals (Japan). Palladium carbon was purchased from E·N Chemcat (Japan). All other chemicals were purchased from commercial sources and used without further purification.

2.2. Synthesis of (D-Asp)$_8$

2.2.1. D-Aspartic acid-β-benzylester (D-Asp(OBzl))

D-Asp (0.1 mol), 80% sulfuric acid (0.1 mol) and benzyl alcohol (0.1 mol) were mixed and dissolved at 70°C. After the mixture was cleared, it was evaporated for 4 h at 70°C. The residue was dissolved in 0.2 M $NaHCO_3$ solution. The crude D-Asp(OBzl) was collected by filtration, then purified by recrystallization from hot water. Yield, 17% (7.2 g) was based on D-Asp (25 g), ^{1}H NMR (D_2O) δ ppm 7.32–7.22 (m, 5H), 5.09 (s, 2H), 3.54–3.50 (t, 1H), 2.92–2.87 (q, 1H), 2.66–2.60 (q, 1H).

2.2.2. N-tert-butyloxycarbonyl-D-aspartic acid-β-benzylester (BOC-D-Asp(OBzl))

To a stirred mixture of D-Asp(OBzl) (9 mmol) and $NaHCO_3$ (18 mmol) in water at 0°C, 12 mmol of (BOC)$_2$O in tetrahydrofuran (THF) was added, and the mixture was continuously stirred overnight at room temperature. The mixture was evaporated, acidified with 10% citric acid, and then extracted with ethyl acetate. The extract was washed with NaCl saturated water and dried with $MgSO_4$. The crude BOC-D-Asp(OBzl) was purified by recrystallization from ethyl acetate–hexane. Yield, 83% (2.4 g) was based on D-Asp(OBzl) (2.0 g), ^{1}H NMR ($CDCl_3$) δ ppm 7.38–7.19 (m, 5H), 5.55–5.53 (d, 1H), 5.15–5.14 (d, 2H), 4.66–4.63 (t, 1H), 3.10–3.05 (q, 1H), 2.92–2.87 (q, 1H), 1.44 (s, 9H).

2.2.3. N-tert-butyloxycarbonyl-D-aspartic acid-β-benzylester-N-hydroxysuccinimide ester (BOC-D-Asp(OBzl)-ONSu)

To a stirred mixture of BOC-D-Asp(OBzl) (12.6 mmol) and HONSu (13.9 mmol) in THF at −20°C, DCC (13.9 mmol) in THF was added, and the mixture was continuously stirred overnight at −20°C. After addition of 1 ml of acetic acid the mixture was stirred at room temperature. The dicyclohexylurea (DCU) was removed by filtration and the THF solution was distilled off *in vacuo*. The residue was dissolved in ethyl acetate, washed with 3% $NaHCO_3$, 3% citric acid and NaCl saturated water, then dried over $MgSO_4$. After evaporation, the crude BOC-D-Asp(OBzl)-ONSu was purified by recrystallization from ethyl acetate–hexane. Yield, 99% (5.27 g) was based on BOC-D-Asp(OBzl) (4.1 g).

2.2.4. N-tert-butyloxycarbonyl-(D-aspartic acid-β-benzylester)$_2$ (BOC-(D-Asp(OBzl))$_2$)

To a stirred mixture of D-Asp(OBzl) (13 mmol) and $NaHCO_3$ (14 mmol) in water, BOC-D-Asp(OBzl)-ONSu (12.5 mmol) in THF was added, and the mixture was stirred at room temperature overnight. The mixture was evaporated and acidified with 10% citric acid and extracted by ethyl acetate. The solution was washed with NaCl saturated water and dried over dry $MgSO_4$. Ethyl acetate was distilled off in vacuo at 30°C. The crude BOC-(D-Asp(OBzl))$_2$ was purified by recrystallization from ethyl acetate-hexane. Yield, 76% (5.0 g) was based on D-Asp(OBzl)-ONSu (5.3 g), ^{1}H NMR ($CDCl_3$) δ ppm 7.54–7.52 (d, 1H), 7.36–7.22 (m, 10H), 5.66–5.65 (d, 1H), 4.87–4.83 (m, 1H), 4.60 (br, 1H), 3.06–2.69 (m, 4H), 1.43 (s, 9H).

2.2.5. *N-tert-butyloxycarbonyl-(D-aspartic acid-β-benzylester)$_2$-N-hydroxysuccinimide ester (BOC-(D-Asp(OBzl))$_2$-ONSu)*

To a stirred mixture of BOC-(D-Asp(OBzl))$_2$ (12.9 mmol) and HONSu (13.5 mmol) in THF at −20°C, DCC (13.5 mmol) in THF was added, and the mixture was continuously stirred at −20°C overnight as described in the synthesis of BOC-D-Asp(OBzl)-ONSu.

2.2.6. *Deprotection of N-tert-butyloxycarbonyl-(D-aspartic acid-β-benzylester)$_2$: preparation of D-Asp(OBzl)$_2$·HCl*

A mixture of BOC-(D-Asp(OBzl))$_2$ (13 mmol) and 4N HCl in dioxane (20 ml) was stirred overnight at room temperature. After evaporation, the reaction mixture was mixed with diethylether, then decanted and dried.

2.2.7. *N-tert-butyloxycarbonyl-(D-aspartic acid-β-benzylester)$_4$ (BOC-(D-Asp(OBzl))$_4$)*

To a stirred mixture of (D-Asp(OBzl))$_2$·HCl (13 mmol) in THF and TEA (28 mmol) at room temperature, BOC-(D-Asp(OBzl))$_2$-ONSu (12.9 mmol) in THF was added, and the mixture was continuously stirred overnight at room temperature as described in the synthesis of BOC-D-Asp(OBzl)$_2$. Yield 3.59 g, ^{1}H NMR ($CDCl_3$) δ ppm 7.66–7.49 (m, 3H), 7.36–7.26 (m, 20H), 5.59 (br, 1H), 5.14–5.08 (m, 8H), 4.94–4.47 (m, 4H), 3.05–2.77 (m, 8H), 1.42 (s, 9H).

2.2.8. *N-tert-butyloxycarbonyl-(D-aspartic acid-β-benzylester)$_4$-N-hydroxysuccinimide ester (BOC-(D-Asp(OBzl))$_4$-ONSu)*

A mixture of BOC-(D-Asp(OBzl))$_4$ (7.5 mmol) and HONSu (8.2 mmol) in THF was stirred at −20°C. After the addition of DCC (8.2 mmol) in THF, the mixture was continuously stirred overnight at −20°C as described in the synthesis of BOC-D-Asp(OBzl)-ONSu.

2.2.9. *Deprotection of N-tert-butyloxycarbonyl-(D-aspartic acid-β-benzylester)$_4$: preparation of D-Asp(OBzl)$_4$·HCl*

A mixture of BOC-(D-Asp(OBzl))$_4$ (8.3 mmol) and 4N HCl in dioxane (20 ml) was stirred overnight at room temperature. After evaporation, the reaction mixture was mixed with diethylether, then decanted and dried.

2.2.10. *N-tert-butyloxycarbonyl-(D-aspartic acid-β-benzylester)$_8$ (BOC-(D-Asp(OBzl))$_8$)*

To a stirred mixture of (D-Asp(OBzl))$_4$·HCl (8.3 mmol) and TEA (28 mmol) in THF at room temperature, BOC-(D-Asp(OBzl))$_4$-ONSu (7.5 mmol) in THF was added, and the mixture was continuously stirred overnight at room temperature as described in the synthesis of BOC-D-Asp(OBzl)$_2$. Yield 4.46 g, ^{1}H NMR ($CDCl_3$) δ ppm 7.66–7.49 (m, 7H), 7.36–7.26 (m, 40H), 5.59 (br, 1H), 5.14–5.08 (m, 16H), 4.94–4.47 (m, 8H), 3.05–2.77 (m, 16H), 1.42 (s, 9H).

2.2.11. *Deprotection of N-tert-butyloxycarbonyl-(D-aspartic acid-β-benzylester)$_8$: preparation of (D-Asp)$_8$*

A mixture of BOC-(D-Asp(OBzl))$_8$ (1.1 mmol) and 4N HCl in dioxane (3 ml) was stirred overnight at room temperature. After evaporation, diethylether was added to the reaction mixture, which was then decanted and dried. TEA was added to a stirred solution of (D-Asp(OBzl))$_8$·HCl in THF. TEA·HCl was removed by filtration and the solution was evaporated. To a stirred mixture of (D-Asp(OBzl))$_8$ in dioxane Pd/C and H_2 gas were added. The mixture was stirred at room temperature for 24 h, then the residual Pd/C was removed by filtration. The filtrate was evaporated, and diethylether was added. Yield 2.0 g, ^{1}H NMR (DMSO-d_6) δ ppm 8.26 (br, 7H), 4.63–3.75 (m, 8H), 2.83–2.66 (m, 16H).

2.3. *Synthesis of (D-Glu)$_8$*

2.3.1. *D-Glutamic acid-γ-benzylester (D-Glu(OBzl))*

D-Glu (0.1 mol), 60% sulfuric acid (0.1 mol) and benzyl alcohol (0.1 mol) were mixed and dissolved at 70°C as described in the synthesis of D-Asp(OBzl). Yield, 66% (16 g) was based on D-Glu (25 g), ^{1}H NMR (D_2O) δ ppm 7.32–7.23 (m, 5H), 4.66 (s, 2H), 4.03–3.99 (q, 1H), 2.26–2.22 (m, 2H), 2.35–2.34 (m, 1H), 1.90–1.87 (m, 1H).

2.3.2. *N-tert-butyloxycarbonyl-D-glutamic acid-γ-benzylester (BOC-D-Glu(OBzl))*

BOC-D-Glu(OBzl) was prepared as described in the synthesis of BOC-D-Asp(OBzl). Yield, 74% (2.1 g) was based on D-Glu(OBzl) (2.0 g), ^{1}H NMR ($CDCl_3$) δ ppm 7.37–7.26 (m, 5H), 5.33–5.31 (d, 1H), 5.12 (s, 2H), 4.25–4.24 (d, 1H), 2.58–2.01 (m, 4H), 1.43 (s, 9H).

*2.3.3. N-tert-butyloxycarbonyl-*D*-glutamic acid-γ-benzylester-N-hydroxysuccinimide ester (BOC-*D*-Glu(OBzl)-ONSu)*

BOC-D-Glu(OBzl)-ONSu was prepared as described in the synthesis of BOC-D-Asp(OBzl)-ONSu. Yield, 92% (2.43 g) was based on BOC-D-Glu(OBzl) (2.0 g).

*2.3.4. N-tert-butyloxycarbonyl-(*D*-glutamic acid-γ-benzylester)$_2$ (BOC-(*D*-Glu(OBzl))$_2$)*

BOC-(D-Glu(OBzl))$_2$ was prepared as described in the synthesis of BOC-(D-Asp(OBzl))$_2$. Yield, 88% (2.7 g) was based on BOC-D-Glu(OBzl)-ONSu (2.4 g), ^{1}H NMR ($CDCl_3$) δ ppm 7.39–7.26 (m, 10H), 5.09 (s, 4H), 4.60–4.55 (q, 1H), 4.27–4.26 (d, 1H), 2.53–1.86 (m, 8H), 1.34 (s, 9H).

*2.3.5. N-tert-butyloxycarbonyl-(*D*-glutamic acid-γ-benzylester)$_2$-N-hydroxysuccinimide ester (BOC-(*D*-Glu(OBzl))$_2$-ONSu)*

BOC-(D-Glu(OBzl))$_2$-ONSu was prepared as described in the synthesis of BOC-D-(Asp(OBzl))$_2$-ONSu.

*2.3.6. Deprotection of N-tert-butyloxycarbonyl-(*D*-glutamic acid-γ-benzylester)$_2$: preparation of *D*-Glu(OBzl)$_2$·HCl*

BOC-(D-Glu(OBzl))$_2$ was deprotected as described in the synthesis of (D-Asp(OBzl))$_2$·HCl.

*2.3.7. N-tert-butyloxycarbonyl-(*D*-glutamic acid-γ-benzylester)$_4$ (BOC-(*D*-Glu(OBzl))$_4$)*

BOC-(D-Glu(OBzl))$_4$ was prepared as described in the synthesis of BOC-(D-Asp(OBzl))$_4$. Yield 2.73 g, ^{1}H NMR ($CDCl_3$) δ ppm 7.69–7.56 (m, 3H), 7.30–7.26 (m, 20H), 5.52–5.44 (m, 1H), 5.08 (s, 8H), 4.59–3.50 (m, 4H), 2.46–1.78 (m, 16H), 1.39–1.36 (m, 9H).

*2.3.8. N-tert-butyloxycarbonyl-(*D*-glutamic acid-γ-benzylester)$_4$-N-hydroxysuccinimide ester (BOC-(*D*-Glu(OBzl))$_4$-ONSu)*

BOC-(D-Glu(OBzl))$_4$-ONSu was prepared as described in the synthesis of BOC-(D-Asp(OBzl))$_2$-ONSu.

*2.3.9. Deprotection of N-tert-butyloxycarbonyl-(*D*-glutamic acid-γ-benzylester)$_4$: preparation of *D*-Glu(OBzl)$_4$·HCl*

BOC-(D-Glu(OBzl))$_4$ was prepared as described in the synthesis of (D-Asp(OBzl))$_4$·HCl.

*2.3.10. N-tert-butyloxycarbonyl-(*D*-glutamic acid-γ-benzylester)$_8$ (BOC-(*D*-Glu(OBzl))$_8$)*

BOC-(D-Glu(OBzl))$_8$ was prepared as described in the synthesis of BOC-D-(Asp(OBzl))$_8$. Yield 11 g, ^{1}H NMR ($CDCl_3$) δ ppm 7.69–7.56 (m, 7H), 7.30–7.26 (m, 40H), 5.52–5.44 (m, 1H), 5.08 (s, 16H), 4.59–3.50 (m, 8H), 2.46–1.78 (m, 32H), 1.39–1.36 (m, 9H).

*2.3.11. Deprotection of N-tert-butyloxycarbonyl-(*D*-glutamic acid-γ-benzylester)$_8$: preparation of (*D*-Glu)$_8$*

BOC-(D-Glu(OBzl))$_8$ was deprotected as described in the synthesis of (D-Asp)$_8$. Yield 4.0 g, ^{1}H NMR (DMSO-d_6) δ ppm 12.05 (br, 1H), 8.32–8.30 (m, 1H), 8.13–8.03 (m, 7H), 4.23–4.04 (m, 8H), 2.36–2.23 (m, 16H), 1.87–1.67 (m, 16H).

*2.4. Screening for (*D*-Glu)$_8$ and (*D*-Asp)$_8$-degrading microorganisms*

(D-Glu)$_8$ and (D-Asp)$_8$-degrading microorganisms were screened from soil samples. The basal medium consisted of 0.2% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract, 1% vitamin mixture solution and 1% trace element solution in tap water, which was adjusted to pH 7.0. The basal medium supplemented with 0.05% (w/v) (D-Glu)$_8$ or (D-Asp)$_8$ was used as an enrichment medium.

The vitamin mixture solution contained biotin (20 mg), calcium pantothenate (4 mg), inositol (20 mg), thiamine·HCl (4 mg), pyridoxine·HCl (4 mg), nicotinic acid (3 mg), *p*-aminobenzoic acid (2 mg), riboflavin (2 mg), and folic acid (100 ml) in 1 l of distilled water.

The trace element solution contained Titriplex III (500 mg), $FeSO_4 \cdot 7H_2O$ (200 mg), $ZnSO_4 \cdot 7H_2O$ (10 mg), $MnCl_2 \cdot 4H_2O$ (3 mg), H_3BO_3 (30 mg), $CoCl_2 \cdot 6H_2O$ (20 mg), $CuCl_2 \cdot 2H_2O$ (1 mg), $NiCl_2 \cdot 6H_2O$ (2 mg), and $Na_2MoO_4 \cdot 2H_2O$ (3 mg) in 1 l of distilled water.

About 100 soil samples, taken from Toyama Prefecture (Japan), were suspended in 10 ml of the enrichment medium in a test tube (∅ 25 mm) and incubated at 30°C on a rotary shaker. Half of the supernatant was removed every other day and the same volume of the fresh medium was added. The consumption of (D-Glu)$_8$ or (D-Asp)$_8$ in the enrichment medium was

subjected to analyses by TLC, in which (D-Glu)$_8$, (D-Asp)$_8$, amino acid oligopeptides and monomers were visualized by spraying ninhydrin.

(D-Glu)$_8$ or (D-Asp)$_8$-utilizing bacteria including *Klebsiella ornithinolytica* EL11, *Delftia acidovorans* DS51, and α-Proteobacteria ES12 were cultured aerobically at 30°C for 48 h in enrichment medium supplemented with 0.1% (w/v) (D-Glu)$_8$ or (D-Asp)$_8$ unless otherwise specified.

2.5. Growth in (D-Asp)$_8$ or (D-Glu)$_8$ containing media

K. ornithinolytica EL11 was cultivated (30°C, 150 rpm) in 5 ml liquid medium as described above, supplemented with 0, 0.1, 0.5, or 1% of either (D-Asp)$_8$ or (D-Glu)$_8$. At regular time intervals an aliquot was taken, optical density (E) at 610 nm was determined (with a Hitachi 1100 spectrophotometer) and dry cell weight was calculated.

2.6. Assay for (D-Asp)$_8$ degradation via HPLC

Culture broth from (D-Asp)$_8$ containing medium was centrifuged at 1700×g for 10 min after 20 h of cultivation. The octamer concentration and appearance of monomer in the supernatant was monitored by HPLC (UV detection at 254 nm; equipped with Sumichiral column, OA-5000 (5 μm) (∅ 4.6 × 1500 mm); Sumika Chemical Analysis Service) at a flow rate of 1.0 ml/min with 2 mM $CuSO_4$.

3. Results and discussion

3.1. Isolation of microorganisms

Ten strains total (five strains from (D-Asp)$_8$ medium, and five strains from (D-Glu)$_8$ medium) were isolated from soil. To obtain growth curves, they were cultivated in liquid medium for two weeks, with either (D-Glu)$_8$ or (D-Asp)$_8$ as the sole source of energy, carbon and nitrogen. Three bacterial strains, EL11, DS51 and ES12, showed good growth on both of the octameric D-peptides and therefore, were identified.

3.2. Identification of microorganisms

3.2.1. Klebsiella ornithinolytica strain EL11

The taxonomical characteristics of strain EL11 are as follows. Rod cells (0.7–0.9 μm × 1.5–3.0 μm), non-motile, non-spore-forming, and Gram-negative. Lysis by 3% KOH: positive. Aminopeptidase (Cerny): positive. Growth on nutrient agar: smooth, entire, convex, round, buff, cream. Anaerobic growth: positive. Gelatin liquefaction, pectin hydrolysis, DNA, or Tween 80: negative. Nitrate reduction: positive. Indole formation: positive. Hydrogen sulfide formation: negative. Acetoin formation (VP) and methyl red: positive. Catalase and urease: positive. Oxidase, tryptophan deaminase, arginine dihydrolase: negative. Ornithine decarboxylase: positive. β-galactosidase and lysine decarboxylase: positive. Phenylalanine deaminase: negative. Citrate utilization: positive. Hugh–Leifson: fermentation. Acid from glucose, fructose, D-xylose, inositol, D-mannose, D-mannitol, maltose, trehalose, L-arabinose, D-sorbitol, L-rhamnose, D-galactose, D-melibiose, adonitol, D-amygdalin, L-arabinose, α-methylglucose, and glycerol. No acid from sucrose, dulcitol, or erythritol. Gas from glucose: positive. The taxonomical characteristics of the strain EL11 indicate that it belongs to the genus *Klebsiella*, because it is rod-shaped, non-motile, oxidase-positive, utilizes citrate, and is VP-positive [15]. Further characterization indicates that it belongs to *Klebsiella ornithinolytica*, because it is ornithine decarboxylase positive [16].

3.2.2. Delftia acidovorans strain DS51

The taxonomical characteristics of strain DS51 are as follows. Short rods (0.7–0.9 μm × 1.5–3.0 μm), motile, non-spore-forming, Gram-negative. Lysis by 3% KOH: positive. Aminopeptidase (Cerny): positive. Growth on nutrient agar: smooth, entire, low convex, opaque. Gelatin liquefaction: negative. Esculin and Casein hydrolysis: negative. Nitrate reduction: positive. Indole formation, esculin hydrolysis, arginine dihydrolase, β-galactosidase: negative. Oxidase and catalase: positive. Urease: negative. Hugh–Leifson: aerobic. Assimilation of carbon compounds: D-mannitol, D-mannose, gluconate, caproate, adipate, DL-malate, citraconate, phenylacetate, m-hydroxybenzoate, and levulinate are assimilated; glucose, L-arabinose, D-mannose, *N*-acetylglucosamine, maltose, and citrate are not

assimilated. Acid from fructose. No growth at 41°C. These taxonomical characteristics indicate that the strain belongs to *Comamonas acidovorans* [17]. Recent studies state that *Comamonas acidovorans* has been removed from the genus *Comamonas* and renamed as *Delftia acidovorans* [18].

3.2.3. α-Proteobacteria strain ES12

The taxonomical characteristics of strain ES12 are as follows. Rods (0.5 μm × 1 μm), non-motile, non-spore-forming, Gram-negative. Lysis by 3% KOH: positive. Aminopeptidase (Cerny): positive. Growth on nutrient agar: smooth, entire, low convex, round, cream. Gelatin liquefaction and casein hydrolysis: negative. Nitrate reduction: negative. Indole formation, esculin hydrolysis, arginine dihydrolase, β-galactosidase: negative. Oxidase and catalase: positive. Urease: negative. Hugh–Leifson: aerobic. No acid and gas from glucose. No acid from xylose. Assimilation of carbon compounds: arabinose, mannose, *N*-acetylglucosamine, DL-malate, and citrate are assimilated; glucose, L-arabinose, D-mannose, D-mannitol, maltose, gluconate, caproate, adipate, phenylacetate, or methanol are not assimilated. Penicillin: insensitive. No growth at 42°C. These taxonomical characteristics of the strain ES12 indicate that it is close to *Moraxella osloensis* [19,20]. However, the results of 16S RNA sequencing indicated that it belongs to a member of α-Proteobacteria [21].

3.3. Assimilation of the D-peptides

Amongst the ten investigated strains, *Klebsiella ornithinolytica* EL11, *Delftia acidovorans* DS51, and α-Proteobacteria ES12, showed best and fastest growth on both of the octameric D-peptides. *K. ornithinolytica* EL11 and α-Proteobacteria ES12 had the highest growth rates on $(\text{D-Asp})_8$, reaching an *E* (at 610 nm) of 0.4 and 0.28, respectively, after 3 days, whereas *Delftia acidovorans* DS51 showed a preference for $(\text{D-Glu})_8$ with *E* (at 610 nm) of 0.3 after 3 days of cultivation. As *K. ornithinolytica* EL11 was the fastest growing strain, it was selected for further investigations. In both media, containing either $(\text{D-Asp})_8$ or $(\text{D-Glu})_8$, the highest growth rate was observed at an octamer concentration of 0.1% (w/v). In the case of $(\text{D-Glu})_8$, this was followed by concentrations of 0.5 and 1%, while in $(\text{D-Asp})_8$-containing medium, growth decreased with concentrations 0.5 and 1%. In the presence of $(\text{D-Glu})_8$ growth proceeded faster during the first 80 h of cultivation, at 0.1% the *E* (at 610 nm) and dry cell weight reached a maximum after 64 h, with 0.83 g dry cells/l. Afterwards, there was a decrease followed by a stagnating phase. Growth with $(\text{D-Asp})_8$ was slower during the early stage of cultivation, a maximum was reached after 170 h, with dry cell weight reaching 1.0 g/l at a $(\text{D-Asp})_8$ concentration of 0.1%. A disappearance of $(\text{D-Asp})_8$ between 10 to 20% was observed, whereas there was an accumulation of monomeric D-Asp, thus indicating that monomers are formed at one stage during the degradation process. Growth of the microorganisms occurred in accordance with the decrease of the substrate.

The new compounds $(\text{D-Asp})_8$ and $(\text{D-Glu})_8$ were synthesized on a large scale by a conventional liquid phase peptide synthetic method utilizing HONSu and DCC [22]. We have successively isolated microbial degraders of these synthetic oligopeptides and confirmed that the D-Asp monomer was formed from $(\text{D-Asp})_8$ during the growth of one of the best degraders *K. ornithinolytica* EL11. Compared with the large amount of literature concerning the biochemistry and enzymology of natural L-peptides, literature concerning D-amino acids containing peptides is scarce [13,14]. It is well known that *Bacillus subtilis* (*natto*) produces extracellular poly-γ-glutamate containing D-glutamate [23], although its biosynthesis and biodegradation are still not well understood [24]. We aimed to screen for enzyme systems other than that of the poly-γ-glutamate degraders, designing our synthetic $(\text{D-Glu})_8$ to have the amide bonds between the 1-carboxylic acid and 2-amino group of D-Glu. This is the first report on the microbial degradation of this type of unnatural D-amino acid-containing oligopeptides. Further studies on the mode of degradation and assimilation of these peptides and the enzyme system responsible for degradation are in progress.

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