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耐熱性カルボニル還元酵素の結晶構造解析と 基質認識機構の解明

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Crystal structure analysis of extremely thermostable carbonyl reductase and
structural basis for substrate recognition

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ABSTRACT

The highly thermostable NAD(P)H-dependent carbonyl reductase (CR; EC 1.1.1.184) from the hyperthermophilic archaeon *Aeropyrum pernix* K1 was crystallized using the sitting-drop vapor diffusion method with L-(+)-tartaric acid (tartrate) as the precipitant. The crystals were found to belong to hexagonal space group $P6_322$, with unit-cell parameters $a = b = 109.8$ Å, $c = 243.7$ Å, $\alpha = \beta = 90^\circ$, and $\gamma = 120^\circ$, and diffracted to 1.80 Å resolution on beamline AR-NW12A at the Photon Factory. The crystal structure of *A. pernix* CR was determined in the presence of NADPH and the substrate analog, tartrate. The ternary complex structure of *A. pernix* CR was determined using the molecular replacement method, and was refined at a resolution of 1.80 Å with a crystallographic R -factor of 22.5% (R_{free} 23.4%). The asymmetric unit consisted of one homodimer. The final structure showed good geometry with no Ramachandran outliers, and consisted of 494 amino acid residues, two NADPH molecules, two tartrate molecules, and 490 water molecules. In the present model, a portion of the surface loop in the C-terminal domain (M192-L197 in subunit A) was disordered and not visible on the electron-density map. Based on the orientation of the tartrate, we modeled the ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] as a product into the active site cavity of the *A. pernix* CR. Within this structure, the hydroxyl group of (*S*)-CHBE forms a hydrogen bond with the side chain of Ser141 and Tyr155. Therefore, in this paper, we describe the structural determination and structure of CR based on the (*S*)-CHBE recognition mechanism and *S*-specific reduction reaction mechanism.

緒 言

カルボニル還元酵素 (CR, EC: 1.1.1.184) はアルデヒド、ケトンあるいはキノンなどの様々なカルボニル化合物の酸化還元反応を触媒する NAD (P) H 依存性の酸化還元酵素である。CR はあまりに基質特異性が広い酵素であるため、生体内において毒物などの生体異物を分解あるいは排泄するための代謝反応に広く関与する酵素であることが推測されているが、本来の生理的意義はほとんど明らかにされていない。これまでに分子量や酵素化学的諸性質が異なる多様な CR が様々な生物種から発見されているにも関わらず (1-4)、全ての CR は短鎖型

脱水素酵素 / 還元酵素ファミリー (SDR ファミリー) に属す。この SDR ファミリーに属す酵素のアミノ酸配列には、触媒において重要な役割を担うチロシン残基が保存されているアミノ酸モチーフ (S-Y_{xxx}K) および酸化還元酵素活性に必須な補酵素 NAD (P) H の結合アミノ酸モチーフ (G_{xxx}G_xG)、の 2 つの共通する配列が厳密に保存されていることが既に知られている (5)。

我々はこれまでにニワトリの脂肪肝特異的に発現する機能未知タンパク質が CR 活性を有する酵素であることや、基質特異性の解析から ethyl 4-chloro-3-oxobutanoate (COBE) を良好な基質として、NAD (P) H 依存的に還元し、ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] を生産する酵素であるという知見を見出している (6, 7)。この反応は産業応用上重要な知見であり、基質 COBE を不斉還元することで、高コレステロール血症治療薬である HMG-CoA 還元酵素阻害剤の原料として有

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用な (S)-CHBE を合成することが可能である (8, 9). また, 本酵素の結晶構造解析にも成功しており, 他の生物種の CR では見られないユニークな基質結合に関わるアミノ酸残基の特定にも成功している (7). しかしながら, ニワトリ脂肪肝由来 CR は3日で酵素活性が半減してしまうほど非常に不安定な酵素であり, 酵素の応用展開には大きな問題があることも判明していた.

そこで, 我々はゲノムデータベースを利用し, ニワトリ脂肪肝由来 CR と相同性を有する好熱菌由来の CR ホモログ遺伝子を検索した結果, 超好熱菌 *Aeropyrum pernix* K1 (最適生育温度95°C) に28%とわずかではあるものの, CR をコードしていると予測される遺伝子 (ORF ID: APE_2503.1) を見出すことに成功した. 本酵素はもともと脂肪酸合成経路に関わる酵素として知られている3-オキソアシル-アシルキャリアプロテイン還元酵素とアノテーションされており, CR 活性を有するのか, また, (S)-CHBE を合成することが可能な酵素であるか全く不明であったが, 酵素のクローニングおよび機能解析を行った結果, 本酵素が高度耐熱性を有する CR であり, また COBE を良好な基質とすることを明らかにしている (10). 超好熱菌由来の CR の報告例はこれまでに無く, 安定性の高い CR は有用なキラル化合物合成などへの有効利用が期待できると考えられる. 本酵素はすでに NADPH との2者複合体の X 線結晶構造解析を行い, NADPH の認識機構や安定化のメカニズムを明らかにしているが, 基質複合体結晶の作成や立体構造解析には至っておらず, 基質認識に関わる詳細な分子メカニズムの情報は不明のままである (10). そこで, 本研究ではこれまでとは異なった晶系の結晶を作成し, 立体構造解析を行うことで *A. pernix* CR-NADPH-酒石酸 (基質アナログ) の3者複合体の構造決定を行うとともに, 基質結合部位に結合していた酒石酸の構造に基づいて CR の反応産物である (S)-CHBE をモデリングすることにより CR の基質認識機構および反応機構を考察した.

材料および方法

試薬

タンパク質結晶化キットである Crystal Screen および Crystal Screen 2は Hampton Research (米国) から購入した. 96穴結晶化プレートは Rigaku (東京) から購入した. L-(+)-酒石酸ナトリウムカリウム四水和物, 及び [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] HEPES はナカライテスク (京都) より入手した. その他の試薬は市販の特級または生化学用試薬を用いた.

耐熱性 CR の発現, 精製および結晶化

超好熱菌 *Aeropyrum pernix* K1由来 CR の大腸菌を用いた発現, 精製は Fukuda らの方法に従って行った (10). MICROCON YM-30 (Millipore, 米国) を使用し精製酵素を5.1 mg/ml まで濃縮後, 還元型補酵素である NADPH および酵素反応のプロダクトである (S)-CHBE をそれぞれ終濃度0.2 mM になるように加え, シッティングドロップ蒸気拡散法を用いて結晶化スクリーニングを行った. すなわち, 96穴結晶化プレートを使用し, 精製酵素 1 μ l とリザーバー溶液 1 μ l を混合した後, リザーバー溶液100 μ l と共に密閉し, 暗所 (20°C) で約1ヶ月静置した.

X 線回折データ測定

得られた結晶を用い, 筑波の高エネルギー加速器研究機構 (KEK AR-NW12A ビームライン) において X 線回折実験を行った. データ測定には気体窒素を吹きかけ, -180°C の極低温条件下で測定を行うクライオ測定の技術を用いた. クライオプロテクタント (抗凍結剤) にはエチレングリコールを選択し, 結晶化条件に終濃度30% (V/V) になるようにエチレングリコール溶液を加え実験に用いた.

X 線の波長は1.00 Å, 振動角度は1イメージにつき1°, 結晶から X 線二次元検出器 (ADSC Quantum 270) までの距離は213.91 mm に設定にした. データの処理には HKL2000 (11) を用いた.

位相決定および構築した構造の精密化

得られた X 線回折データを元に分子置換法を用いて, *A. pernix* 由来 CR の NADPH-基質アナログ3者複合体の位相決定を行った. 分子置換のプログラムには MOLREP (12) を用いた. サーチモデルには *A. pernix* 由来 CR-NADPH の2者複合体の立体構造データ (PDB code = 5B1Y) を用いた.

プログラム COOT (13) を用いて1-250アミノ酸残基をアミノ酸配列通りに電子密度にフィッティングを行った. その後, プログラム REFMAC5 (14) および CNS (15) により構造の精密化を行った. *A. pernix* CR の立体構造の妥当性 (ペプチド結合の2面角の妥当性; ϕ , ψ 角) の評価は MOLPROBITY (16) を用い, ラマチャンドラプロットで解析を行った. (S)-CHBE の座標ファイルは ChemDraw (HULINKS Inc.) および PRODRG サーバー (<http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrgr/>) を用いて作成した. 水素結合の検出にはプログラム

CCP4mg (17) を用い、分子構造の描画にはプログラム PyMOL (DeLano Scientific LLC.) を用いた。

結果および考察

結晶化およびデータ測定

結晶化条件のスクリーニングを行ったところ、Crystal Screen No. 29 (0.1 M HEPES バッファー pH 7.5, 0.8 M L-(+)-酒石酸ナトリウムカリウム四水和物) の条件での単結晶の析出が確認できた。更に、沈殿剤である酒石酸の濃度を0.4-1.0 M の範囲で条件展開することで約0.3

mm のバイピラミッド型の単結晶を作成することに成功した (Fig. 1A)。本結晶を用いて X 線回折実験を行ったところ、最高分解能1.80 Å であり、空間群は六方晶系である $P6_122$ であった (Fig. 1B)。すでに報告している *A. pernix* 由来 CR-NADPH の 2 者複合体 (PDB code = 5B1Y) の解析に用いた結晶は2.0 M NaCl を沈殿剤として得られた結晶であり、最高分解能が2.09 Å、空間群 $P4_12_12$ であったことから、本研究ではこれまでとは異なった晶系の結晶作成および、より高分解能なデータ収集を行うことができた。

立体構造解析および全体構造

解析の結果、活性中心には水以外の分子と予測される明瞭な電子密度が観察されたため、クライオの測定溶媒に用いたエチレングリコール分子と予測して、電子密度にアサインしたものの、エチレングリコールが2分子分以上入る大きさの電子密度であり、カルボキシル基と予測される形状の電子密度も観察された。そこで、結晶化試薬として高濃度使用した酒石酸の存在が予測された

Table 1. Data-collection, processing, phase determination, and refinement statistics for *A. pernix* CR.

Data collection	
Wavelength (Å)	1.00
Temperature (K)	100
Space group	$P6_122$
Unit cell parameters (Å, °)	$a = b = 109.8, c = 243.7$ $\alpha = \beta = 90, \gamma = 120$
Resolution range (Å) ^a	50.00–1.80 (1.83–1.80)
No. measured reflections	2307616
No. unique reflections	80994
Redundancy ^a	28.5 (29.6)
R_{merge} ^a	0.080 (0.307)
$R_{\text{p.i.m.}}$ ^a	0.016 (0.061)
Completeness (%) ^a	99.9 (100)
$\langle I/\sigma(I) \rangle$	13.1
Refinement	
Resolution range (Å)	35.5–1.80
R/R_{free} ^b	0.225/0.234
No. of protein atoms	3735
No. of water molecules	490
No. of ligands	NADPH, 2 Tartaric acid, 2
RMSD	
Bond lengths (Å)	0.025
Bond angles (°)	2.3
Average B-factors (Å ²)	
Protein atoms	21.0
Water molecules	36.9
NADPH molecules	44.1
Tartaric acid molecules	41.3
Ramachandran quality ^c	
Favored regions (%)	96.5
Outliers (%)	0.0

^a Values in parentheses are for the last resolution shell.

^b R_{free} was calculated with randomly selected reflections (5%).

^c MOLPROBITY (16) was used to monitor and validate the structural model.

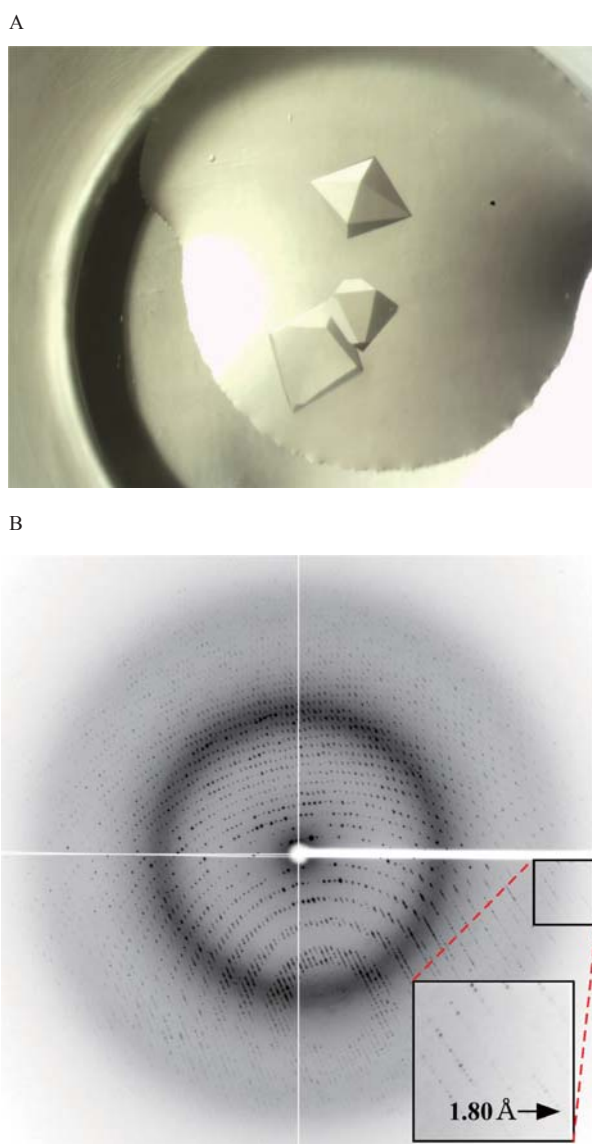


Fig. 1. Hexagonal crystals and X-ray diffraction pattern of *A. pernix* CR. (A) *A. pernix* CR/NADPH/tartrate ternary complex at 1 month in the crystallization step using the sitting-drop vapor-diffusion method at 20°C. The maximum dimensions of the crystal were 0.3 × 0.3 × 0.1 mm. (B) The X-ray diffraction pattern of a native *A. pernix* CR crystal. The resolution is approximately 1.80 Å at the edge. The high-resolution area is enlarged (inset).

め、電子密度にアサインしたところ、ぴったり当てはまり、活性中心に観測された電子密度を酒石酸として解釈することができた。また、NADPHは電子密度へ容易にフィットすることが可能であった。最終的に *A. pernix* 由来 CR-NADPH-酒石酸の3者複合体を R 値 =

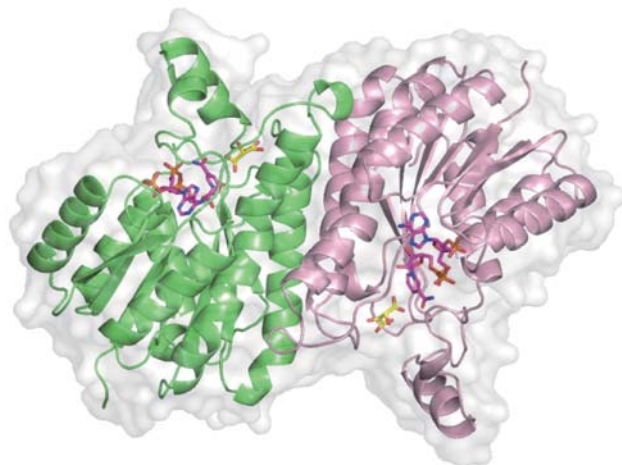


Fig. 2. The dimeric structure of *A. pernix* CR. The subunit A and B are shown in green and pink, respectively. NADPH (magenta) and tartrate (yellow) molecules are shown as a stick model. Oxygen and nitrogen atoms are shown in red and blue, respectively.

0.225, free R 値 = 0.234で構造決定することに成功した (Table 1). 非対称単位中にはホモダイマーが1分子存在しており、494アミノ酸残基、2分子のNADPH、2分子の酒石酸、490の水分子で構成されていた (Fig. 2). また、マッシュアップ係数は $3.49 \text{ \AA}^3 \text{ Da}^{-1}$ 、結晶中の溶媒含有量は64.5%であった。MOLPROBITYを用いたラマチャンドラプロットの解析の結果、全てのアミノ酸残基が許容範囲内であった。モノマー構造はロスマンフォールドドメイン (N末ドメイン) と触媒ドメイン (C末ドメイン) の2つで構成されていた。

BサブユニットはMet1-Thr250の250アミノ酸残基全てでモデル構築することができたが、Aサブユニットの分子表面のループ領域であるMet192-Leu197の6アミノ酸残基は明瞭な電子密度が観察できず、アミノ酸をアサインすることができなかった。また、本酵素はpET-15b (Novagen, 米国) をタンパク質発現用プラスミドとして使用しているため、His-tag配列 (20アミノ酸) がCRのN末端に付加されているが、構造解析の結果、His-tag配列の電子密度は観察されなかった。結晶化の際にCRの酵素反応プロダクトである (*S*)-CHBEと共結晶化したものの、結晶化の沈殿剤として使用した高濃度の酒石酸 (0.8 M) が基質結合部位を占有し、(*S*)-CHBEは基質結合部位に結合しなかったと考えられる。

酒石酸複合体構造および (*S*)-CHBE のモデリング

活性中心に結合している酒石酸のC1カルボキシル基は触媒残基であるSer141, Tyr155の側鎖と水素結合しており、酒石酸のC4カルボキシル基はArg248の側鎖およびVal150, Ala151の主鎖のNHと水素結合していた (Fig. 3A). また、酒石酸のカルボニル基はArg248の側

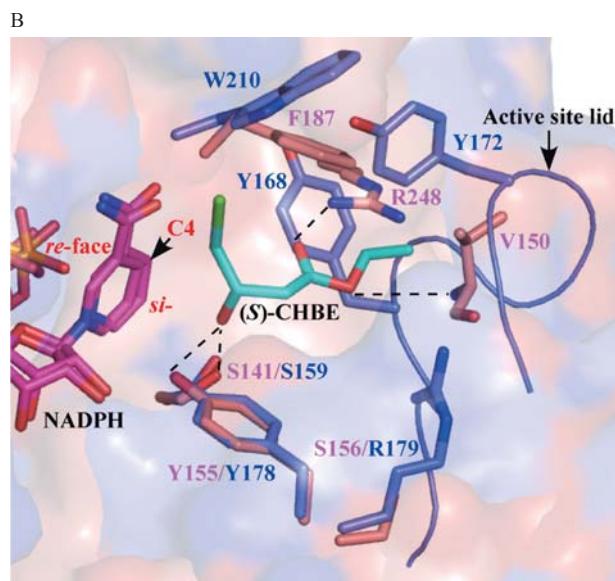
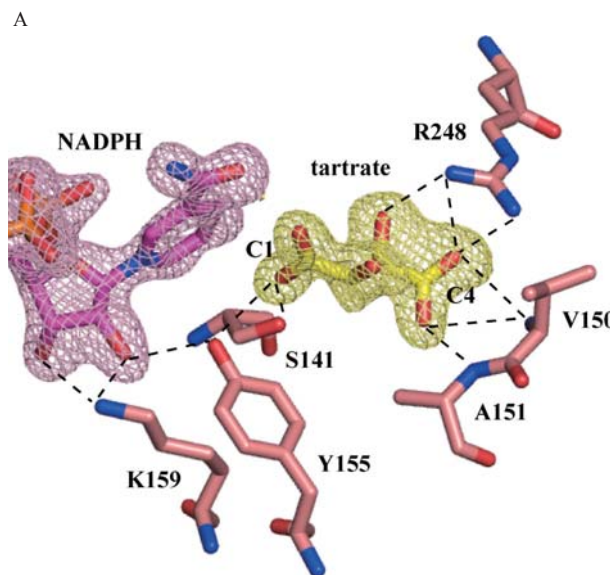


Fig. 3. The tartrate coordinated within the active site and the proposed binding model for the (*S*)-CHBE molecule. (A) Schematic representation depicting the interactions between tartrate and *A. pernix* CR. The networks of hydrogen bonds are shown by dotted lines. The tartrate molecule is shown as a stick model in yellow. The final σ_A -weighted F_o-F_c omit electron density map for tartrate is shown at the 1σ level. (B) Comparison of the *A. pernix* CR active site pocket with that of chicken fatty liver CR and the proposed binding model of the (*S*)-CHBE. The C4 atom of the pyridine ring (a hydride acceptor site), and *si*- and *re*-faces are labeled. NADPH (magenta) and (*S*)-CHBE (cyan) molecules are shown as a stick model. The structures of *A. pernix* and chicken fatty liver CR are shown in pink and blue, respectively. Oxygen, nitrogen and chloride atoms are shown in red, blue and green, respectively.

鎖と相互作用することを明らかにした。触媒残基である Lys159は NADPH のニコチンアミドリボースのヒドロキシル基との水素結合していた (Fig. 3A)。得られた酒石酸複合体の構造から C1カルボキシル基の部分が CR の基質のカルボニル基の位置に相当するのではないかと予測されたため、活性中心に結合した酒石酸 (基質アナログ) の配向から CR の酵素反応産物である (S)-CHBE の結合モデルを構築し、これまで不明であった基質結合に関与するアミノ酸残基を同定した。モデリングの結果、(S)-CHBE のヒドロキシル基は触媒残基である Ser141, Tyr155の側鎖と水素結合しており、(S)-CHBE のカルボニル基は Arg248の側鎖と水素結合していた (Fig. 3B)。また Val150が (S)-CHBE の酸素原子および疎水性のエチル基と相互作用することが予測され、S 体選択的に反応が可能な配向で (S)-CHBE が *A. permix* 由来 CR の触媒部位に結合することが示唆された (Fig. 3B)。補酵素 NADPH のニコチンアミドリボース間の結合が *syn* 型の構造であることから NADPH のピリジン環の C4位からの水素転移は *si* 面 (*pro-S*; B-type) 特異的に起こると考えられることも S 体選択的に COBE の還元反応が可能な理由と言える。

A. permix CR とニワトリ脂肪肝 CR の構造比較

これまでの我々の研究により *A. permix* 由来 CR およびニワトリ脂肪肝由来 CR の両酵素において COBE が良好な基質となることをすでに明らかにしている (7, 10)。COBE に対する親和性はニワトリ脂肪肝由来 CR の方がはるかに高い事をすでに報告している (*A. permix* CR; $K_m=0.7\mu\text{M}$, ニワトリ脂肪肝由来 CR; $K_m=0.008\mu\text{M}$) が、その分子認識機構は未だに不明であった。そこで、(S)-CHBE のモデリングを行った *A. permix* CR の構造とニワトリ脂肪肝由来 CR (PDB code = 3WXB) の重ね合わせを行い、活性中心に結合した (S)-CHBE の周辺構造を比較することで、基質に対する親和性の違いについて考察した。*A. permix* CR では (S)-CHBE のエチル基とクロル基の周辺に存在するアミノ酸は Val150, Phe187, Arg248であるが、ニワトリ脂肪肝由来 CR では (S)-CHBE の疎水性のエチル基を疎水性アミノ酸である Tyr168, Tyr172が認識するとともに、マイナスにチャージしているクロル基と Trp210の NH がインタラクションし、(S)-CHBE の酸素原子は構造上、Arg179がインタラクトすることが推定された (Fig. 3B)。ニワトリ脂肪肝 CR の Tyr168, Tyr172は基質結合部位にフタをする役割の “Active site lid” (Ile164-Gln174) 内に存在するアミノ酸残基であり、他の生物種の CR ではアミノ酸残基の

保存性が全くないユニークな配列である。特に、Y172 に関してはアラニン置換変異体の解析により、基質である 9,10-phenanthrenequinone に対する K_m 値が12.5倍上昇することをすでに明らかにしている (7)。モデリングの結果では Y172が活性中心で (S)-CHBE を抑え込むような形でインタラクションすることが予測されることから、モデリングした構造と酵素化学的データの結果が良く一致していると言える。これらの結果より、ニワトリ脂肪肝由来 CR では *A. permix* CR と比較して (S)-CHBE とのインタラクションがより強固になっていることを立体構造に基づき説明することが可能となった。これらの結果は、高脂血症治療薬の合成中間体として有用なキラル化合物である (S)-CHBE に対するハイアフィニティーな酵素の創製などへの応用利用に役立つ情報となりうる。

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要 約

カルボニル還元酵素 (CR, EC: 1.1.1.184) は NAD (P) H を補酵素として様々なカルボニル化合物の還元反応を触媒する酵素である。安定性の高い CR は有用なキラル化合物合成などへの有効利用が期待できる。本研究では超好熱菌である *Aeropyrum permix* K1由来の耐熱性 CR の NADPH-酒石酸 (基質アナログ) との3者複合体の結晶構造解析を行うとともに、CR の酵素反応産物である (S)-4-クロロ-3-ヒドロキシ酪酸エチル [(S)-CHBE] の構造を活性中心にモデリングした。0.8 M L-(+)-酒石酸を沈殿剤として NADPH-酒石酸との複合体結晶を取得し、X 線回折実験を行った結果、最高分解能1.80 Å のデータを取得し、最終的に分子置換法により *A. permix* CR-NADPH-酒石酸の3者複合体の構造を決定することに成功した。触媒部位では Ser141, Tyr155が酒石酸の C1カルボキシル基との水素結合に関与し、Lys159は NADPH のニコチンアミドリボースのヒドロキシル基との水素結合に関与していた。酒石酸の構造に基づいて

(*S*)-CHBE のモデリングを行ったところ、*S* 体選択的に反応可能な配向で (*S*)-CHBE が基質結合部位に結合することが示唆された。これら構造情報は、高脂血症治療薬の合成中間体として有用なキラル化合物である (*S*)-CHBE 生産のための人工 CR 創製への応用利用に役立つ情報となりうる。

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***Lactobacillus plantarum* LA 318 inhibits the adhesion of *Candida albicans* ATCC 26555 to human colonic mucin**

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ABSTRACT

Candida albicans is a fungal organism found in small numbers in the normal human intestinal tract. Normally kept in check by the body's own helpful bacteria, *C. albicans* can increase in numbers when this balance is disturbed to cause candidiasis of the intestinal tract, or yeast infections in other parts of the body. Here an adhesion inhibition of the yeast and pseudohyphal forms of *C. albicans* ATCC 26555 to human colonic mucin by *Lactobacillus plantarum* LA 318 was investigated. SDS-PAGE showed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at about 30 kDa in *Candida albicans* ATCC 26555 using SYPRO Ruby stain while few bands were detected using CBB stain. Forms incubated at 28 ° C (induces yeast) and 37 ° C (induces hyphae) showed a dose-dependent adherence of *C. albicans* ATCC 26555 to human colonic mucin. Adhesion inhibition tests were performed using three different methods: competition, exclusion, and displacement assays. Adhesion of the yeast form incubated at 28 ° C was significantly decreased by about 10 ~ 20 % ($p < 0.05$) ; whereas no inhibition of fungus adhesion was observed in the competitive assay using 1×10^8 cells/mL of LAB (equal to fungus numbers) . The highest inhibition was observed using the exclusion assay with 1×10^9 cells/mL of LAB (10 fold higher than the *C. albicans* ATCC 26555) . Adhesion of the pseudohyphal form incubated at 37 ° C when using equal numbers of LAB was slightly decreased; whereas the pseudohyphal form adhesion was significantly decreased by 21.5 % in the exclusion assay using 1×10^9 cells/mL of LAB ($p < 0.05$) . This study shows the possible prevention of candidiasis using lactobacilli.

INTRODUCTION

Candida species are the most frequent cause of life-threatening invasive fungal infections in immunocompromised hosts (1). *Candida* colonization often leads to opportunistic mucosal or deep organ infection in immunocompromised hosts. Invasion of the human gastrointestinal mucosa by *C. albicans* and its passage across the bowel wall into the bloodstream is an important portal of entry, leading to systemic or disseminated candidiasis (2-5). Adhesion of the fungus is an important first step in the infection of the host. It has been reported *C. albicans* ATCC 26555 adheres to the

extracellular matrix (6-8) ; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is expressed on its cell surface (7).

Lactic acid bacteria (LAB) are common microbes used as probiotics. The genus *Lactobacillus* shows many beneficial effects as probiotics, e.g. managing lactose intolerance (9), lowering cholesterol (10), improving immune function (11), prevention of colon cancer (12), and inhibition of adherence of pathogens (13-15). *Lactobacillus plantarum* LA 318 is a potential probiotic strain isolated from a normal human intestinal tissue (transverse colon) of a cancer patient and strongly adheres to human colonic mucin (HCM) and blood type antigens (A type and B type) mediated by a cell surface

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GAPDH (16, 17). The adhesion of *C. albicans* ATCC 26555 may be inhibited by *L. plantarum* LA318 through a competitive interference, because both the *Candida* and LAB strains possess GAPDH on their cell surface.

C. albicans is dimorphic, having a yeast form and a pseudohyphal form, according to the temperature environment. Pathogenicity is shown when in pseudohyphal growth (18–20). An adhesion inhibition test to HCM using *L. plantarum* LA 318 with the two different forms of *C. albicans* ATCC 26555 was investigated.

MATERIALS AND METHODS

Microorganisms and growth conditions

L. plantarum LA 318 was directly isolated from human colon tissue using a mass screening. This strain showed high adhesion to HCM and A and B blood type antigens by cell surface GAPDH (16, 17). The strain was cultured twice at 37 °C for 24 h in MRS broth (Difco Laboratories, Detroit, MI, USA) using a 2% (v/v) inoculum.

C. albicans ATCC 26555 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). This strain was reported to have GAPDH on the cell surface (7). The strain was cultured twice at 28 °C for 24 h in YM broth (Difco Laboratories) using a 2% (v/v) inoculum.

For the inhibition assay, *C. albicans* ATCC 26555 was cultured for 16 h at 28 °C. The cells were centrifuged (4,000 rpm, 10 min) and the supernatant was discarded. The pellets were held for 3 or 4 days at 4 °C (starvation period). After starvation, fresh YM broth was added and fungi cells were cultured for 6 h at either 28 °C or 37 °C.

Extraction and analysis of surface proteins of *C. albicans*

C. albicans ATCC 26555 were propagated for 16 h at 28 °C and washed three times with PBS (PBS wash solution). After washing, the cells were gently suspended in 2 or 4 M guanidine hydrochloride (GHC1) solution and incubated at 37 °C for 2 h. After centrifugation (8,000 rpm, 20 min, 4 °C), the supernatant (extract) was used for the GAPDH activity assay. The PBS wash solution and GHC1 extract were dialyzed against distilled water and lyophilized. The lyophilized extracts were used for SDS-PAGE.

SDS-PAGE

The cell surface extracts of *C. albicans* ATCC 26555 were

analyzed using SDS-PAGE in a 12.5 % gel. After electrophoresis, protein bands were visualized staining the gels with Coomassie brilliant blue (CBB) (Rapid CBB KANTO, Kanto Chemical Co., Tokyo, Japan) and SYPRO Ruby (BIO-RAD Laboratories, Hercules, CA, USA).

Carboxyfluorescein diacetate labeling of *Candida* cells

After the starvation period, *C. albicans* ATCC 26555 was cultured at 28 °C or 37 °C for 6 h in YM broth (Difco Laboratories) and centrifuged (5,000 rpm, 10 min, room temperature). The pellets were washed with sterilized distilled water three times (8,000 rpm, 5 min, 4 °C). The pellets were re-suspended in sterilized distilled water and 1 mM carboxyfluorescein diacetate (CFDA) was added in PBS. After incubation for 30 min at 37 °C in the dark, the CFDA labeled microbial cells were washed with sterilized distilled water three times (8,000 rpm, 5 min, 4 °C).

Microtiter plate binding assay

Reacti-Bind™ amine-binding maleic anhydride activated plates (PIERCE, Rockford, IL, USA) that allow attachment of amine-containing protein to the microplate wells were used for the microtiter plate binding assay. The isolation and purification of HCM was performed as described previously (21). The purified HCM (0.3 mg/mL) was dissolved in PBS (pH 7.4) (100 μL) and added to each well and incubated overnight at 4 °C; and then the plates were used for the binding assay. After immobilization of the HCM, each well was washed three times with 250 μL PBS. Then the CFDA-labeled *C. albicans* ATCC 26555 were added in 100 μL distilled water to the wells. Three parallel wells were used in each experiment. The microbial cells were allowed to adhere for 1 h at 37 °C and the wells were washed three times with 250 μL distilled water to remove non-adherent cells. The cells bound to the HCM were released and lysed using 1% (w/v) SDS/0.1 M NaOH solution and incubated for 1 h at 60 °C. After incubation, the fluorescence intensity (Ex: 485 nm, Em: 538 nm) of the lytic solution was measured using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). PBS was used as the control substituting for HCM. The adhesion value was defined as the value of HCM-*Candida* immobilized on the plate subtracting the control value.

Adhesion inhibition test for *C. albicans*

The adhesion inhibition tests were performed using three

different methods: competition, exclusion, and displacement assays. The concentration of CFDA-labeled *C. albicans* ATCC 26555 was 1×10^8 cells/mL. The concentrations of non-labeled *L. plantarum* LA 318 were 1×10^8 cells/mL or 1×10^9 cells/mL.

For the competitive assay, each 50 μ L of CFDA-labeled fungus and 50 μ L of non-labeled LAB strain was mixed and added to the HCM-immobilized microtiter wells; and incubated for 1 h at 37 $^{\circ}$ C. Each well was washed three times with 250 μ L distilled water and the fungus cells bound to the HCM were released and lysed using 1% (w/v) SDS/0.1 M NaOH solution and incubated for 1 h at 60 $^{\circ}$ C. After incubation, the fluorescence intensity (Ex: 485 nm, Em: 538 nm) of the lytic solution was measured using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Inc.).

For the exclusion assay, 100 μ L of non-labeled LAB was added to the HCM-immobilized microtiter wells and was incubated for 1 h at 37 $^{\circ}$ C and washed three times. After washing, 100 μ L of CFDA-labeled fungus was added and incubated for 1 h at 37 $^{\circ}$ C. After washing, the cells were lysed and fluorescence intensity was measured.

For the displacement assay, the LAB and fungus were reversed in the exclusion assay.

Statistics

All experiments were performed in triplicate and reported as the mean \pm SD. Statistical analyses of all data were performed using the Scheffe's test for the adhesion test and the Tukey's HSD (honestly significant difference) test for the inhibition assays.

RESULTS AND DISCUSSION

In this study, inhibition of adherence of *C. albicans* to human intestinal mucus using a high adhesive probiotic LAB, *L. plantarum* LA 318 was considered. It is reported that *C. albicans* ATCC 26555 possess surface GAPDH and shows adhesion to fibronectin and laminin as the fungal pathogen (7). *C. albicans* ATCC 26555 was propagated at two difference temperatures, 28 and 37 $^{\circ}$ C, after starvation. The fungus morphed into yeast cells at 28 $^{\circ}$ C or pseudohyphal cells at 37 $^{\circ}$ C (Fig. 1). Pathogenicity is increased during pseudohyphal elongation

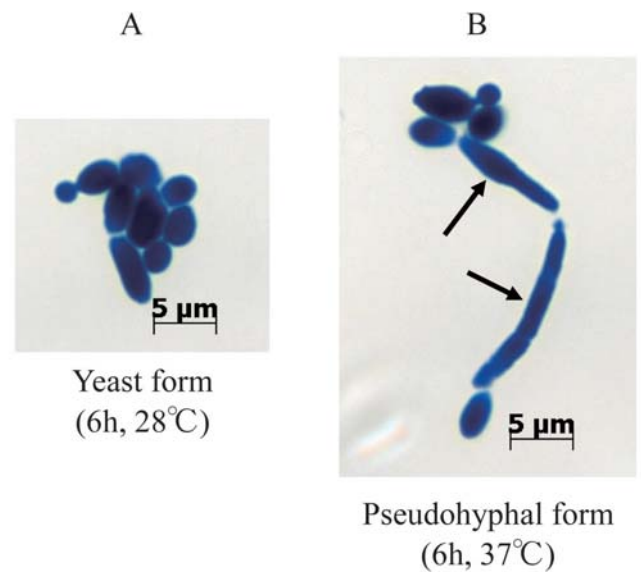


Figure 1. The two different forms of *C. albicans* ATCC 26555 using incubation at different temperatures (28 $^{\circ}$ C or 37 $^{\circ}$ C). After the starvation period, *C. albicans* ATCC 26555 was cultured at 28 $^{\circ}$ C (A) or 37 $^{\circ}$ C (B) for 6 h in YM broth. Each of cells were stained with Gram's stain and observed with microscope. Arrows indicate a germ tube.

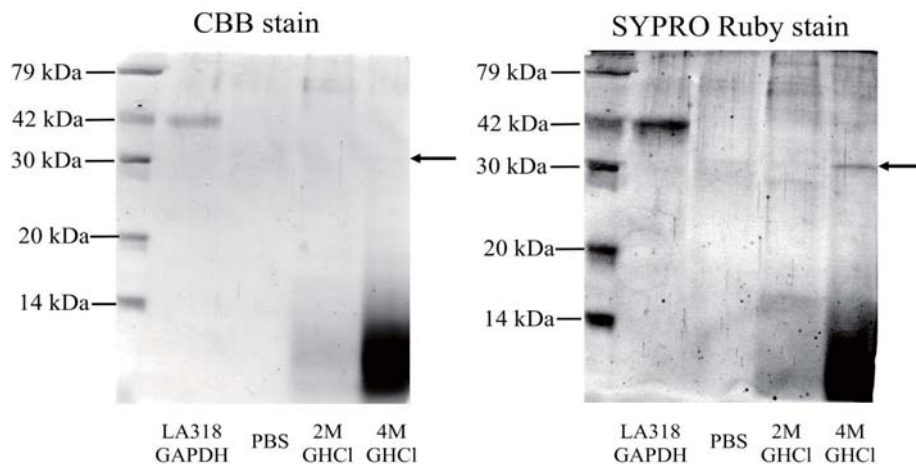


Figure 2. SDS-PAGE of protein extracts from *C. albicans* ATCC 26555. LA 318 GAPDH: purified GAPDH from *L. plantarum* LA 318, PBS: PBS wash fraction, 2M GHCl: 2 M GHCl extracts, 4M GHCl: 4 M GHCl extracts.

(18–20). Figure 2 shows the SDS-PAGE of cell surface extracts of *C. albicans*. A 30 kDa protein was detected using SYPRO Ruby stain while few bands were detected using CBB stain. This suggested the 30 kDa protein was GAPDH because the molecular weight of GAPDH from *C. albicans* ATCC 26555 is 33kDa. Further, GAPDH enzymatic activity in the extract was detected. However, the expression level was very low compared to the *L. plantarum* LA 318 (data not shown). Therefore, GAPDH may be not the primary adhesin of *C. albicans* ATCC 26555 where other adhesins may exist. It was reported that a 58 kDa fibronectin-binding mannoprotein (mp58) was expressed on the *C. albicans* cell surface as an adhesion (6) but it was not detected. Since a lot of protein was detected at ≤ 14 kDa, the possibility of an adhesion factor at < 14 kDa was considered.

Reports show some pathogens possess GAPDH on their cell surface and they are identified as adhesins. Streptococcal surface GAPDH (SDH) from group A *Streptococcus* shows multiple binding activities to plasmin (ogen) (22, 23), fibronectin, lysozyme, myosin, and actin (24). SDH recognizes uPAR/CD87 as its receptor on human pharyngeal cells and mediates bacterial adherence to host cells (25). Seifert *et al.* report enzymatically active GAPDH is capable of binding to cytoskeletal and extra-cellular matrix proteins and is expressed on the surface of group B *Streptococcus* (26). Surface-localized GAPDHs of *Staphylococcus aureus* and *Staphylococcus epidermidis* bind transferrin (27); whereas the GAPDH of *C. albicans* binds fibronectin and laminin (7). The fimbriae of *Porphyromonas gingivalis*, a dominant periodontal pathogen, mediates co-agglutination using the GAPDH of *Streptococcus oralis* (28) to bind to human oral epithelial cells (29). Tunio *et al.* report the GAPDH (GapA-1) of *Neisseria meningitidis*, causing life-threatening infections including septicemia and meningitis, adhered to human cells (30). Therefore, many pathogenic bacteria possess GAPDH on their surfaces that show binding to multiple tissues.

In studies of lactobacilli, Hurmalainen *et al.* report GAPDH is expressed on cell surfaces with enolase in seven lactobacilli and one lactococci. The proteins bound plasminogen (Plg) and enhanced its activation using a tissue-type Plg activator (tPA) (31). Antikainen *et al.* report GAPDH bound to lipoteichoic acid on the bacterial cell surface using ionic bonds (32). GAPDH, elongation factor Tu (EF-Tu) and triosephosphate isomerase (TPI) are

surface-bound proteins that play a role in adhesion of *L. plantarum* 423 to Caco-2 cells (33). The strain prevented *Clostridium sporogenes* and *Enterococcus faecalis* from adhering to Caco-2 cells. Sánchez *et al.* report GAPDH and phosphoglycerate kinase are expressed on the cell surface of *L. rhamnosus* GG (34). Castaldo *et al.* report *L. plantarum* LM3 expresses enolase on the cell surface bound to fibronectin, an extracellular matrix protein (35). We previously reported cell surface GAPDH of *L. plantarum* LA 318 showed high adhesion to HCM and ABO blood type antigens, especially A $\{ \text{GalNAc } \alpha 1-3 (\text{Fuc } \alpha 1-2) \text{ Gal-} \}$ and B $\{ \text{Gal } \alpha 1-3 (\text{Fuc } \alpha 1-2) \text{ Gal-} \}$ antigens where GAPDH may be a GalNAc and Gal recognition lectin-like protein (16–17). Further, cell surface GAPDH activities were detected from 30 lactobacilli isolated from human intestinal tissues. A statistically significant positive correlation was shown between GAPDH activity and adhesion using the BIACORE adhesion assay (0.69, $p < 0.01$) (36).

Figure 3 shows the *C. albicans* ATCC 26555 adhesion test to HCM using microtiter plates. Both 28 °C and 37 °C incubated cells adhered to HCM. The amount of *C. albicans* ATCC 26555 adhesion increased in a dose-dependent manner. The detection limit was 1×10^7 cells/mL for the yeast form (28 °C) and 1×10^6 cells/mL for the pseudohyphal form (37 °C). *C. albicans* ATCC 26555 was reported to adhere to fibronectin (6–7), laminin (7), and the 7S (IV) domain of type IV collagen (8); and other *C. albicans* strains adhered to human buccal epithelial cells, rabbit's small intestinal mucin (37), and saliva mucin (38, 39). In this study, *C. albicans* ATCC 26555 shows adhesion to HCM.

The adhesion inhibition tests were performed using three different methods: competition, exclusion, and displacement assays. Adhesion of the yeast form incubated at 28 °C were significantly decreased by about 10 ~ 20 % ($p < 0.05$); whereas no inhibition of fungus adhesion was observed in the competitive assay when using 1×10^8 cells/mL of LAB (equal to fungus numbers) (Fig. 4A). The highest inhibition was observed in the exclusion assay when using 1×10^9 cells/mL of LAB (10-fold higher than *C. albicans* ATCC 26555). Adhesion of the pseudohyphal form incubated at 37 °C when using equal numbers of LAB was slightly decreased; whereas the pseudohyphal form adhesion was significantly decreased by 21.5 % in the exclusion assay when using 1×10^9 cells/mL

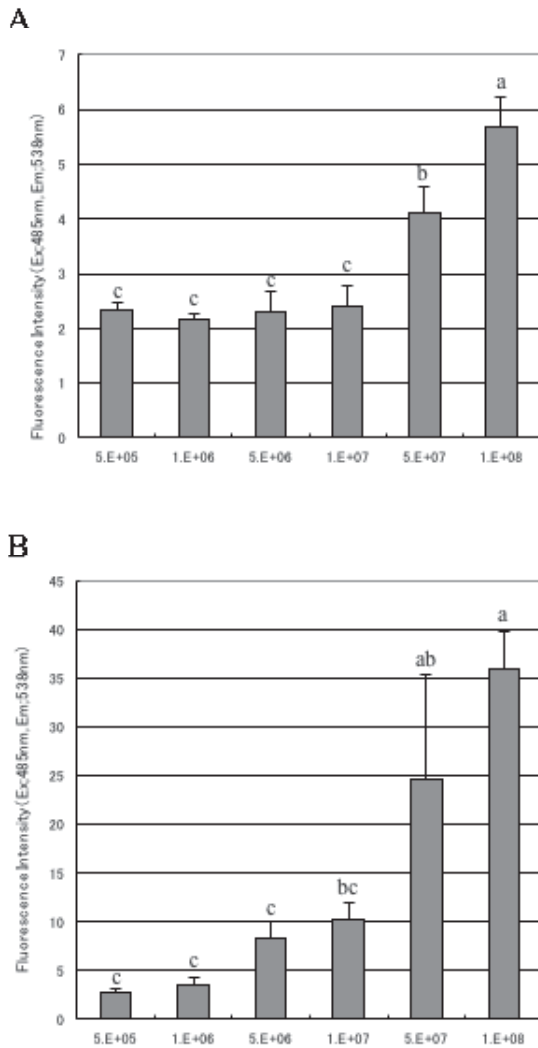


Figure 3. Adhesion test for *C. albicans* ATCC 26555 to HCM using the microtiter plate assay. A: *C. albicans* ATCC 26555 incubated at 28 °C , B: *C. albicans* ATCC 26555 incubated at 37 °C . Different letters show significant difference ($p < 0.05$).

of LAB ($p < 0.05$) (Fig. 4B). The difference in the amount of LAB and *Candida* cells may be one cause. The cell size of *C. albicans* ATCC 26555, a eukaryote, is ten times larger than LAB, a prokaryote. When the concentration of LAB was 1×10^8 cells/mL (equal to *C. albicans* ATCC 26555), LAB occupied a smaller volume of the well. When concentration of LAB was 1×10^9 cells/mL (10-fold higher), the occupied LAB volume of the well was equal to *C. albicans* ATCC 26555. LA 318 showed adhesion to HCM in a dose-dependent manner at from 1×10^7 cells/mL to 1×10^{10} cells/mL in the microtiter plate binding assay (data not shown). If LAB cells are added 100 times or more as much as *C. albicans* ATCC 26555 cells, there may be further inhibition. It is well known indigenous bacteria protect a human body from various pathogens including *C. albicans*. The ingestion of LA 318

may help indigenous bacteria's protective effects.

Reports show the inhibitory effect on candidiasis by LAB strains. Boris *et al.* report *L. acidophilus* inhibited adhesion of *C. albicans* and *Gardnerella vaginalis* to vaginal epithelial cells in competition and displacement assays; whereas there was no inhibition using an exclusion assay (40). Coudeyras *et al.* report *L. rhamnosus* Lcr35 adhered to cervical and vaginal cells and inhibited the growth of vaginosis-associated pathogens such as *C. albicans* (41). Matsubara *et al.* report lactobacilli reduced the *C. albicans* colonization significantly in the oral mucosa compared to untreated mice (42). In the group treated with *L. rhamnosus*, yeast colonization was significantly reduced compared with that of the group receiving nystatin. The effect of *L. casei* CRL 431 administered as a supplement to a repletion diet was studied for resistance of malnourished mice to *C. albicans* infection (43). The addition of *L. casei* to the repletion diet normalized the immune response against *C. albicans*, allowing efficient recruitment and activation of phagocytes, as well as effective release of pro-inflammatory cytokines. Martinez *et al.* reported *L. reuteri* RC-14 alone or together

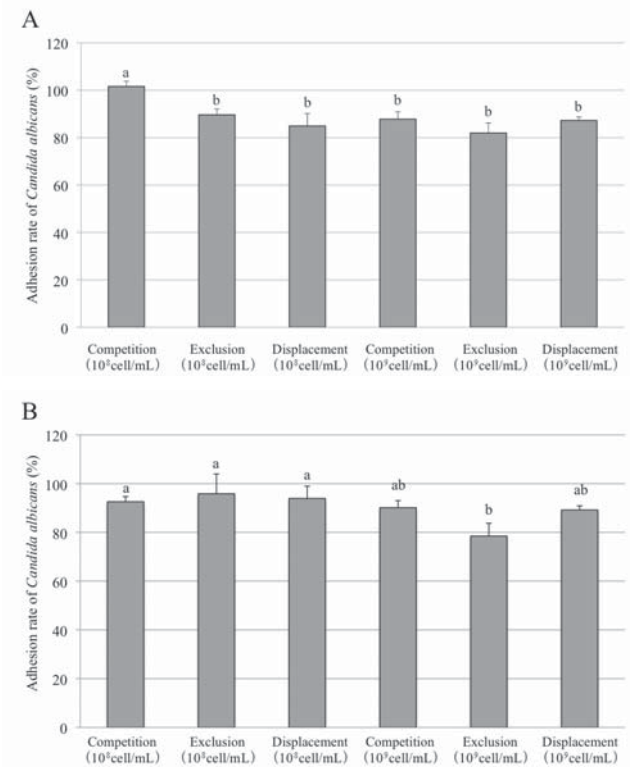


Figure 4. Adhesion of *C. albicans* ATCC 26555 to HCM in the inhibition assay with *L. plantarum* LA 318. A: using *C. albicans* ATCC 26555 incubated at 28 °C , B: using *C. albicans* ATCC 26555 incubated at 37 °C . Different letters show significant difference ($p < 0.05$).

with *L. rhamnosus* GR-1 has the potential to inhibit yeast growth, and their cell-free supernatants may up-regulate IL-8 and IP-10 secretion by epithelial cells from the normal human vagina that may possibly play an important role in clearing vulvovaginal candidiasis (44). Further, LAB produces antifungal substances such as lactic acid and hydrogen peroxide (H₂O₂) (45, 46). Therefore, there are potential benefits against *Candida* infection using LAB.

CONCLUSION

In this study, the adhesion inhibition to HCM by *L. plantarum* LA318 using yeast and pseudohyphal forms of *C. albicans* ATCC 26555 was investigated. SDS-PAGE showed GAPDH in *C. albicans* ATCC 26555 while it is suggested that GAPDH is not a primary adhesin because few bands are detected using the CBB stain. In the adhesion inhibition tests, the inhibition of *C. albicans* ATCC 26555 adhesion to HCM using the probiotic LAB, *L. plantarum* LA 318, especially with the exclusion assay was found. The exclusion assay can be regarded as preventative. Therefore we believe a highly adhesive LAB may be used for the prevention of candidiasis. This study shows the possibility of prevention of candidiasis using lactobacilli.

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Lactobacillus plantarum LA 318はヒト大腸ムチンへの *Candida albicans* ATCC 26555の付着を阻害する

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要約

Candida albicans はカンジダ症を引き起こす日和見感染菌である。*C. albicans* は培養条件により酵母型と仮性菌糸型の二形成を示す。本試験ではヒト大腸ムチン (HCM) に高い付着性を示す *Lactobacillus plantarum* LA 318を用いて *C. albicans* ATCC 26555の付着阻害の可能性を検討した。SDS-PAGEにより *C. albicans* ATCC 26555の菌体表層タンパク質中にグリセルアルデヒド-3-リン酸脱水素酵素 (GAPDH) の発現を確認したが、その発現量は非常に少なかった。このことから GAPDH は、*C. albicans* ATCC 26555のメインとなるアドヘシンではない可能性が考えられた。また、*C. albicans* ATCC 26555は酵母型、仮性菌糸型ともに HCM に対し菌数依存的な付着性を示した。カンジダの付着阻害試験は、競合、排除、置換試験の3つのパターンで行った。28℃培養のカンジダ菌 (酵母型) では、 1×10^8 cells/mL の乳酸菌の競合阻害以外は10~20%の付着阻害が見られた ($p < 0.05$)。最もカンジダ菌の付着阻害が見られたのは 1×10^9 cells/mL の乳酸菌を用いた排除試験だった。37℃培養のカンジダ菌 (仮性菌糸型) では、 1×10^8 cells/mL の乳酸菌を用いた試験においては、競合、排除、置換試験のいずれにおいても僅かな阻害しか示さなかったが、 1×10^9 cells/mL の乳酸菌では排除試験において21.5%のカンジダ菌の付着の減少が見られた ($p < 0.05$)。これらの結果より乳酸菌を用いた *C. albicans* の感染阻害の可能性が示された。

Exploring Academic English for the New Curriculum at Tokai University

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Summary

In this paper the authors outline the steps taken to prepare for the development of a new class to be called “Academic English” for the upcoming curriculum change at Tokai University. The definition of Academic English for the School of Agriculture, what exactly is meant by Academic English in the context of the Tokai University School of Agriculture, is presented and methods are suggested. A review of pertinent literature on related topics and methods was done. Based on the findings in the literature review, a pilot class was conducted in the fall semester of 2015. The outcome of the pilot class was positive, but it was also noted that the self-selected participants of the pilot class were more motivated and better in English than the average student in the School of Agriculture. This made clear the need for more thought on the development of materials for the new class. The inclusion of Academic English in the new curriculum is an opportunity to start a tailor made English educational program at the School of Agriculture with the cooperation of academic teachers. This change is hoped to lead to better opinions toward studying English, better understanding of why students should study English, and better performance in their study of English.

Introduction

Background

At the Tokai University School of Agriculture, a new English curriculum will start in 2018. Eight credits of English classes are required at present and that will stay the same. As for the subjects, there will be two new ones, Academic English and Global Skills. These will replace the current Reading and Writing 2 and Listening and Speaking 2. These changes will produce a new English curriculum which consists of four subjects, namely; Listening and Speaking, Reading and Writing, Global Skills, and Academic English. Consistent with the current class offerings, Academic English and Global Skills will both be two credits and class will meet twice a week for 15 weeks. The definition of Academic English provided by the Tokai University Educational Council was lacking in detail, but the council suggested that English language instructors cooperate with instructors of the academic subjects in the development and teaching of the Academic English classes. The first step in this cooperation

was a questionnaire to the teachers in the agriculture department. A review of the relevant literature was conducted, and subsequently a pilot class was conducted in the fall semester of 2015. The outcome of the pilot class was positive, but this was tempered by the fact that the self-selected participants of the pilot class were generally higher in both motivation and English level than the average student in the School of Agriculture. These differences in motivation and English ability between the pilot class and the general student population made clear the need for further consideration of the choice and adaptation of materials for the new class.

Questionnaire to Academic Teachers

The Tokai University Educational Council presented a new subject, Academic English, however, the definition was not provided in detail. No New Paragraph The subject was described in broad strokes with instructions to cooperate and discuss the details with the School of Agriculture. As part of the development of the new Academic English class, the original plan was to have English teachers interview the faculty in the School of Agriculture. However, due to various logistical considerations, information was collected by the

authors by means of a questionnaire given to the agriculture teachers. There are three departments within the School of Agriculture, Bio Science, Plant Science, and Animal Science. In the present curriculum, each department has two classes titled "Reading Academic Papers in English." All agricultural students are expected to take these classes in which the focus is to read academic papers written in English. In the questionnaire, the academic teachers were asked what types of materials were used in those classes. Among the teachers responding to the questionnaire, sixteen said the students read academic papers, ten said they read overseas textbooks, six said they read general science books, four said they read English textbooks for junior and senior high school students and university liberal arts level students. With regard to the question of what agriculture teachers want the students to learn in Academic English, the responses were grammar, translation, vocabulary, how to use a dictionary and web translation, and academic writing. When asked about the skills that are necessary for academic study besides Reading Academic Papers in English, the responses were oral communication, writing, and listening to lectures. As to the question "How much can you cooperate with English teachers in Academic English classes?", seven indicated they would like to give advice to curriculum development and material selection, six said it was difficult to cooperate, three responded that they would be able to help students as follow-up to Academic English, but no one wanted to participate in the teaching of Academic English classes.

From the questionnaire result it became clear that the academic teachers do not seem to be content with the present English education at the School of Agriculture, wondering what the students learn in the first two years of university English classes. By the time students have completed the required eight credits of English, the agriculture teachers expect the students to have mastered basic English grammar and vocabulary, and to be able to read basic science passages. The faculty want to know why, after six years of junior and senior high school and then two years at university studying English, students still have not mastered these basic English skills. One reason is that the students can graduate junior and senior high school without learning English. The students then expect the same to hold true at university. They feel that English is not necessary for study or for daily life. They are not interested or confident in learning English.

Definition of Academic English

In order to be able to develop a curriculum with an Academic English class, one should be clear about what exactly is meant by "Academic English". A review of relevant literature provided valuable insight into several important considerations. For example, it is important to consider the relationship between language classes and content classes (1). In order to investigate what methods are currently employed by English teachers to teach English using content relevant to students, the field of Teaching English to Speakers of Other Languages was researched. It became clear that as Brinton *et al.* (2) state, "The claim that language is most effectively learned in context is hardly a new or revolutionary one." Immersion education started in the 1960's in Canada (3) and the United States, however, it has been found that learning content in a second language was taking place about 5000 years ago in the area which is modern-day Iraq. At that time, it was the Sumerians teaching theology, botany and zoology to the conquering Akkadians using Sumerian (4, 5). The focus on learning the content while learning the target language came naturally. Content-Based Instruction (CBI), Language across the Curriculum, Language for Specific Purposes, Content and Language Integrated Learning (CLIL), and Task-Based Language Teaching (TBLT) are the same in the sense that all combine language learning and content learning. However, there can be differences in the focus and the actual methods used (6, 7).

For example, Immersion focuses on content and by studying the content students learn a target language. More specifically, immersion uses the learners' second language (L2) as the medium of classroom instruction. In an immersion classroom, students study school subjects, such as algebra, biology, and history, in their L2. The main purpose of this method is to promote bilingualism. This incorporates the development of learners' communicative competence or language proficiency in their L2 in addition to their first or native language (L1). "Intensive exposure to the target language through natural communication with a native speaker was considered essential, as was starting at a young age (2)." Due to this fact, this method has most commonly been found in programs for primary or secondary school students.

Content-based instruction (CBI) is a form of communicative language teaching. In CBI, language instruction is integrated with academic content instruction. CBI implies the total

integration of language learning and content learning (8). The intention behind CBI is to provide second-language learners instruction in content and language. In this context, content is taken to mean the use of subject matter as a vehicle for second or foreign language teaching/learning. In CBI, it is expected that learners acquire language together with content through the awareness-raising activities the teacher makes use of. For this reason, the learner is expected to process language consciously as well as intuitively. In the case of CBI, teachers are required to familiarize themselves with difficult and unfamiliar content and often need to develop their own courses or choose and adapt materials that provide a basis for CBI (9).

As for CLIL the focus is on both a target language and content (10–12). “The term ‘Content and Language Integrated learning’ (CLIL) was adopted in 1994 (Marsh, Maljers and Hartiala, 2001) within the European context to describe and further design good practice as achieved in different types of school environment where teaching and learning take place in an additional language (13).” This methodology focuses on supporting language within context. Language teachers are teaching through a language rather than in another language. This serves to assist learners in acquiring both language and content as they learn a school subject (12). Both CBI and CLIL require the learners to actively participate, and share the goal of moving toward learner autonomy.

As Academic English will be taught by English teachers, the focus is more on language. Therefore, CBI would seem to be an appropriate model for Academic English. There are three types of CBI. One is Theme-Based Language Instruction, another is Sheltered Content Instruction, and the third is Adjunct Language Instruction (2). Sheltered Courses are content courses taught in the second language to a group of learners by a content area specialist. In the adjunct model, students are enrolled concurrently in two courses, a language course and a content course. These two courses share the content base and complement each other in terms of mutually coordinated assignments. The use of theme or topic-based language courses is one way to increase the use of subject matter content in language classes. In such courses the language class is structured around topics or themes, with the topics forming the backbone of the course curriculum. The language teacher provides the basis for language analysis and practice through the content material presented (2). In the case of the Tokai University School of Agriculture, Academic

English can be defined as theme or topic-based language courses which seem most appropriate as the English teachers will not have much support by content teachers as the questionnaire results show.

Topics and Materials of Academic English

An academic English class is not a class geared toward reading academic papers in English class, therefore English teachers should not utilize the same methods such as reading academic papers, American high school biology textbooks, or general science books in English. English teachers are not biology or agriculture teachers. Therefore, they are not responsible for teaching content. However, as intelligent, responsible global citizens, English teachers are interested in current topics related to agriculture, such as the water crisis, virtual water, ecological footprint, food mileage, GMO, and organic food. They have access to such information in English, and that is what they can use in the Academic English classes. By studying this information, the students will have more knowledge of the topic while learning the language used to discuss it. English teachers are not required to teach the content. English teachers should be able to rely on academic teachers to explain in detail any content or concepts related to the subject matter that they are not familiar with. English teachers can encourage students to consult with academic teachers or English classes can have guest speakers. However, it should be noted that the questionnaire results do not show much eagerness on the academic teachers' side.

The material used for CBI should be authentic (2). However, considering the English level of the students, English teachers cannot use the material as it is. Brinton *et al.* (2) bring up the need to adapt authentic materials. In “Reading Academic Papers in English” classes, academic teachers are expected to use authentic materials. Therefore, it is a good stepping stone to use adapted authentic material in Academic English to work as a bridge to Reading Academic Papers in English.

Skills of Academic English

Choosing good tasks is the key to the success of CBI. Stryker (8) introduces sheltering activities, such as lectures, small- and large-group activities, student projects and presentations, invited speakers, and the use of a variety of audio-visual aids. Academic teachers expressed in the questionnaire that searching the internet in English is a

necessary skill. Also, not only reading, but also writing, listening, and speaking are important. Critical thinking is the base of all these skills. The sheltering activities Stryker introduced above are similar to the skills academic teachers think are important. The following are the kind of activities that can be employed:

- (1) Listening to mini lectures
- (2) Reading
- (3) Internet search
- (4) Oral presentation with power point, discussion
- (5) Writing

Language of Academic English

17 out of 21 academic teachers commented in the questionnaire that they want students to have basic vocabulary and grammar. A command of grammar and basic vocabulary are necessary to read academic papers, but there is a concern about classes centered around grammar and vocabulary. The students had such classes at junior and senior high school and repeating the same type of lessons does not seem to be a preferable method. Mohan (1) says, "Both in research and in classroom practice it makes little sense to disconnect language learning from learning about the world. There is no reason for research to be restricted to the study of language learning in isolation from content learning and from contexts of communication. There is no reason for the language classroom to be restricted to teaching language for its own sake." In a class of German students studying English, Brinton *et al.* (2) chose a theme-based model stating that, "The theme-based model was selected as an effective way of providing a meaningful context for refining the English language skills of advanced students. Since the German students had been exposed extensively to English grammar and vocabulary in their high school English classes, a curriculum designed around a grammatical syllabus would have been very repetitious. In addition, the program designers hoped that the lagging motivation of advanced students could be revitalized in a theme-oriented course which capitalizes on what the students already know and fills in the gaps in a contextualized manner." This brings us back to "The claim that language is most effectively learned in context is hardly a new or revolutionary one (2)." Stryker (8) says, "However, many of us who, in our initial enthusiasm for CBI, minimized or removed the formal study of grammar, found that our adult students wanted – and needed – to deal with grammar in an

explicit, deductive manner." Therefore, although Academic English should be a topic-based language course and not a grammar- or lexical- based language course, that does not mean the elimination of teaching grammar. When it is necessary to focus on form, it should be done.

A Preliminary Pilot Study

Class

To get some insight on how the Academic English suggested above would work, in the fall semester of 2015 a small-scale pilot class was conducted. The class was in the first period on Tuesday, when the first-year Class 2 and the second-year Class 1 did not have English classes as the first-year Class 1 and the second-year Class 2 had English classes at that time. First, a flyer was created to introduce students to a pilot study for the Academic English class. The flyer was handed to all the first-year Class 2 and the second-year Class 1 students. There were about 250 students. The pilot class met once a week for 9 weeks.

Participants

Eight students participated in the pilot class and on average five of them participated every week. Their placement English scores ranged from 38 to 66 out of 100. One of them was a Master's degree student. Four of the undergraduate students were in the top class.

Material

The material from New Internationalist Easier English Wiki (14) was used. There were such topics as food waste, cowspiracy, seeds of change.

Teaching Method

As this was a pilot class conducted with self-selected participants from among the students in the School of Agriculture, reasonably high levels of motivation were expected. The volunteers did display good motivation and interest in the topics. Even so, it was felt that there should not be too great a demand placed on students to work on the pilot class material outside of the class time. Materials were prepared ahead of time and distributed during the class. Generally, a "packet" of material included various reprints of reading material, vocabulary and grammar exercises, comprehension questions, and discussion points relating to the

topic. While the readings were short, being only 2 to 6 pages in length on average, there were often several related readings for a single topic. Therefore, this material was of sufficient volume to allow the same packet to be used over a period of several classes. The students were allowed, but not required, to take the material home between class sessions to study at their leisure.

Since students were not required to do any outside preparation, a portion of class time was devoted to reading and comprehending the material. As the material was geared toward non-native speakers of English, the grammar structures were generally familiar to the students. Vocabulary provided a challenge, as many of the words used in the material were specific to the topic. Although students were slightly familiar with the topics, they had almost exclusively been exposed to the material in Japanese until the time of the pilot class.

The class was very much teacher centered. Students received English language input from both the instructor and the reading materials provided. Language output from the students was almost entirely verbal as no written assignments were given. The only writing was in the form of short answers to some comprehension and discussion questions. While the lack of written output might be considered less than ideal for an academic English class, for the purposes of this pilot study it was felt to be acceptable.

Questionnaire Results

A questionnaire was conducted after each class. There were a total of 42 responses from students who participated in the pilot class. The following are the results.

Table 1. Content of material

Content of material	Number of student responses
Very interesting	40
Fairly interesting	2
Not so interesting	0
Not interesting at all	0

Table 2. Difficulty of English

Difficulty of English	Number of student responses
Very easy	0
Quite easy	3
Just right	20
A little difficult	18
Very difficult	1

Table 3. Familiarity with content

Familiarity with content	Number of student responses
Very familiar	0
Fairly familiar	13
Not so familiar	27
Not familiar at all	2

The level of the English seemed adequate, however, the English level of the participants was quite high. Given the fact that there will be students with lower English proficiency taking Academic English, adaptation of this easier English to an even easier level will be necessary. Next, although the participants were agriculture students, they were not overly familiar with the topics. One of the possible reasons was that the participants were mainly first year. However, there was one graduate student who participated, and he also said that the English was difficult and that he was not familiar with the topic. It seems that what the students learn in academic classes is not related to our everyday life or what is happening in the present world. The research is more particular, scientific, and advanced.

Conclusion

This paper describes the steps taken in the development of Academic English for the School of Agriculture. A review of pertinent literature on related topics and methods was done. Based on the findings in the literature review, Academic English was defined as theme or topic-based language courses, methods and materials were determined, and a pilot class was conducted in the fall semester of 2015. The outcome of the pilot class was positive. However, the need for more thought on the development of materials for the new class was made clear.

Suggestions for 2018

The students of the Academic English pilot class voluntarily participated with a high level of attendance. That fact, in addition to the questionnaire results, make it evident that the students generally had positive attitude toward the class. It should be noted, however, that four out of seven undergraduate students were in the top class, therefore, the students who will be required to take this Academic English class would likely have lower English proficiency and be less

motivated. One thing that can be done to overcome this is to adapt the pilot class English material to make it even easier. Another possibility is to increase cooperation between the English and academic teachers. It should not be that English teachers teach English and then send the students into academic classes. Instead, the academic teachers should come in at an earlier stage and emphasize the necessity of English. The CBI approach to language learning, the blending of language and content, would help to motivate the students. The focus is placed on content and not on grammar or general reading. This shift in focus makes it different from the English classes they had at junior and senior high school.

The idea of Academic English was imposed by Tokai University Educational Council. It is, after all, a great opportunity to start a tailor made English educational program at the School of Agriculture with cooperation of academic teachers. It is hoped that the students will finally master the English skills that they will need in their academic classes.

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東海大学新カリキュラムのアカデミック英語の開発に向けて

ロドリゲス八木美樹

Daniel Minor

抄録

東海大学農学部では2018年度から新英語カリキュラムが始まり、アカデミック英語が新設される。まず、農学部教職員に対して行ったアンケート調査の報告として現カリキュラムの外書講読で扱っている教材、アカデミック英語で学生に学習してほしい内容、外書講読以外に専門分野の学習に必要な英語スキルが挙げられた。又、アカデミック英語への協力は限定的になることと現英語カリキュラムには不満をもっていることが明らかになった。次に、内容と言語の両方に焦点をあてた様々な教授法を紹介する。そして、それを基に農学部でのアカデミック英語の教授法を提案する。具体的には農学分野のトピックの一般的なリーディング、短い英語での講義のリスニング、インターネットを使っての調査、パワーポイントを使った口頭発表、議論、まとめのライティングである。高校までの英語教育の繰り返しにならず、学生の興味・関心を引くように内容中心の授業になるが必要に応じて文法や語彙の指導も行う。2015年度秋学期にはアカデミック英語の試行授業を行った。その参加者、教材、教授法、参加者に対して行ったアンケート調査を報告する。希望者に対するものであったので当然、英語力、意欲はもともと高いが、結果を見るとアカデミック英語に対して殆どが肯定的な感想をもっていた。最後に2018年度開始に向けた提案と改善案として、実際は試行授業の参加者より英語力も意欲も低いので、教材の内容は変えずに英語を更に平易にすることと農学部教員が専門の勉強には英語が必要であることを早い段階で示す必要性を挙げている。英語教員と農学部教員との連携により学生の英語力向上が可能になると考える。

Recommended Modifications to the Kambara Locus of Control Scale

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Summary

This paper reports on the results of a mixed-methods approach towards the evaluation of a psychometric instrument commonly used in the Japanese research context for measuring the construct known as locus of control (LoC), namely the Kambara Locus of Control Scale (K-LoC). Kambara (1987) created this 43-item scale in order to measure LoC in Japanese high school students. With the goal of verifying whether the K-LoC actually measures what it purports to measure, the author conducted a confirmatory factor analysis (CFA) with an a priori set of data collected for this purpose. The dimensionality of the scores produced by the instrument were found to not be structurally valid, indicating unsatisfactory model fit for the original conception of the instrument (Rupp, 2016a). Based on this outcome, the author subsequently conducted a focus group study in order to provide a qualitative perspective from the Japanese university student population with which to further inform the results of the CFA (Rupp, 2016b). The results of the focus group study revealed numerous potential problems with the scale such as issues with the Likert scale, item content and survey length. In order to further explore the scale, the author decided to conduct an exploratory factor analysis (EFA) which added further explanatory power to the previous two studies, particularly with regard to item factor loadings (Rupp, in press). Taking into account the previous research on the K-LoC, this paper, applying a sequential explanatory mixed-methods approach (Cresswell, 2014), represents the combined analysis of the EFA and focus group studies, with the goal of elucidating changes which are recommended be made to the K-LoC in order to remedy the deficiencies outlined in the previous studies.

The Locus of Control Construct

This study is situated in a larger research context of attempts at measuring learner autonomy through notionally related constructs, such as attribution theory (e.g. Heider, 1958; Kelley, 1967; McLeod, 2010), self-efficacy theory (Bandura, 1977), self-determination theory (Deci & Ryan, 1985) and locus of control theory (Rotter, 1966). The LoC construct refers to a unidimensional continuum, ranging from internal to external, for which people are supposed to fall upon to the degree to which they believe that outcomes in their lives are controlled by internal factors (I-LoC) or external factors (E-LoC). Oxford (2003, 2008) posits that having a higher degree of I-LoC corresponds to possessing higher degrees of agency and self-efficacy, which are key components of learner autonomy.

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Language Learning and Locus of Control

Ghonsooly and Moharer (2012), conducted a Persian version of the Duttweiler (1984) LoC (5-point Likert Scale) to investigate the correlation between I-LoC and translation students' achievement. Using statistical analysis on data gathered from university seniors in the English translation department (N=151), they found a significant positive relationship, which was further backed up by results from a qualitative interview phase of the study. In another study (Ghonsooly & Elahi, 2010), using the 28-item (Duttweiler, 1984), five-point Likert scale, a significant positive relationship was found between I-LoC and general English achievement among university students (N=240), as well as finding differences in I-LoC levels among groups of students based on their major fields of study (humanities, sciences, and engineering). Studying the relationship between LoC and L2 reading and writing achievement with university English

major students in Iran (N=136), using the Internal Control Index (ICI) created by Duttweiler (1984), Ghonsooly and Shirvan (2011) found a positive correlation between I-LoC and higher achievement in reading and writing. They conclude that teachers can empower students by applying attribution theory through reattribution training to help students view their failures as being due to controllable factors such as effort, thus moving them from E-LoC orientations to I-LoC orientations, and thus enabling students “to become autonomous L2 readers or L2 writers” (p. 238).

A study conducted in Taiwan (Chang & Ho, 2009) using Crandall’s (Crandall, Crandall, & Katkovsky, 1965) I-E LoC instrument with university students not majoring in English found a positive correlation between I-LoC and comprehension as well as self-efficacy using a web-based interactive instructional system with two types of control: learner and program. The highest scores on the test were received by students who had both a high I-LoC orientation, as well as those who learned from the learner-control version of the learning environment.

In recent research exploring the relationship between learner autonomy and the language learning locus of control in multilinguals, Peek (2016) used data from an online language learning beliefs survey (N=842) to define language learning experience as it related to the participants’ multilingual capacities. Using statistical analyses, they found a significant positive correlation between multilingualism and higher degrees of I-LoC in a domain specific study of language learning of locus of control (LLLOC). Specifically, they found that language learners might be more autonomous learners when they subscribe to beliefs that align with a more internal LLLOC.

The Kambara Locus of Control Scale

The K-LoC (see Appendix A) emerged as a prominent scale used in the Japanese research context in a wide range of domains over the last 30 years, including employee psychology (Fushimi, 2011), developmental psychology (Kanda, 2006), education (Kambara, 1987) and English language achievement (Hosaka, 2007). The K-LoC was originally developed in a short (K-LoC18) 18-item form (Kambara, Higuchi, & Kiyomizu, 1982) which was later expanded (K-LoC43) to 43 items (Kambara, 1987), and it is the K-LoC43 which is the scale which was found to be predominantly used in Japan. Based on the author’s previous

studies conducted in Japanese on a Japanese high school population, both the K-LoC18 and K-LoC43 were found to produce scores which did not support model fit according to CFA (Rupp, 2016a) analysis. This result was followed by a focus group study involving Japanese university students to analyze the K-LoC43 (Rupp, 2016b) the outcomes of which highlighted many potential problems with the K-LoC43. These outcomes are analyzed below in light of a subsequent EFA (Rupp, in press) conducted by the author. This combined analysis leads to a summary of changes which are recommended to be made to the K-LoC for future use in the Japanese research context.

Summary of Focus Group Outcomes

This section will summarize the themes which arose in the focus group discussions (Rupp, 2016b) about the K-LoC43. Appendix A shows a translation of the K-LoC43 along with the tabulation of votes by focus group members on item appropriateness for the university level. This tabulation was conducted as the K-LoC43 was originally designed for high school students, in order to find items which would need to be modified or eliminated for university student measurement studies. The general feedback on the K-LoC43 from the 27 focus group members is found in Appendix B and the item specific comments are found in Appendix C.

Individual survey general criticisms and comments included criticisms about the length of the survey, occasional unnatural Japanese language usage, difficult item content, an unexpected Likert scale ordering, an overly rough scale range, age/life-stage inappropriate items and item similarity. There were also positive comments including praise of a unique and thought provoking items which surprised some students and caused others to engage in deep self-reflection.

Regarding the numerous complaints about survey length and repetitious nature of some items, it should be noted that the original Kambara LoC Scale (1982) was only 18 items, referred to here as the K-LoC18. Kambara expanded the scale in order to focus more on items related to high school life. While the students experienced these kinds of questions as redundant, there is in fact a measurement rationale for measuring a construct from both a positive and negative frame as a means of addressing response set bias and issues of item skew. Nevertheless, based on the students’ comments in this study, it appears that such a shorter scale would be preferable

or that more care is needed in constructing the negative and positive items so that they are not merely inverse statements. This is supported by the results of the EFA as will be discussed further below. The reduced implementation time required for an instrument with fewer items scale would be an additional benefit.

A common complaint was that it was felt to be impossible to form an opinion about certain items, often due to the content being perceived as more appropriate to an older, more established working adult. Thus many students were forced to choose either 2 or 3 (on a 4-point Likert scale, corresponding to a “slightly disagree” or “slightly agree” answer) even though the real answer would have been a 3 on a five-point Likert scale (corresponding to a “neither agree nor disagree” answer). This desire for a more refined Likert scale was reiterated by the fact that many students expressed an explicit desire for a middle choice, such as would be available on a 5-point Likert scale, due to the fact that they felt they could not give accurate answers when there was truly no preference. This can be a tricky issue as some researchers feel it is important to force a choice, rather than leaving an easy middle answer which can be chosen with less serious consideration. In this case more nuanced results might be possible with a 6-point scale.

There were a few students who indicated that in their experience Likert scales generally start from 1 being a positive answer to 4 (or 5) being a negative answer, although this scale used the original Kambara (1987) scale going from negative to positive. In other words, many students felt that it was more common for the positive response to begin on the left, and the negative part of the scale to be on the right. Though not a universal sentiment, students who pointed this out appeared to feel quite strongly about it.

Another common complaint was that the intentions and/or assumptions underlying some of the questions were unclear, causing frustration and test fatigue. Regarding the Japanese, although some students indicated there were no problems with the Japanese, other felt that some items should be reworded, often by making the question sound less extreme by adding an “I think...” before the question. There were also a number of students who expressed frustration at not understanding the goals of the survey and how it could relate to English education. Other students found questions about making friends with the opposite sex to have an unnecessary emphasis on gender.

Summary of EFA Results for K-LoC43 and K-LoC18

The EFAs used data collected from 1125 high school students from three local high schools, two private and one public, from all three grade levels with 57% male and 43% female participants. The data was analyzed using IBM/Statistical package for the Social Sciences (SPSS) software Version 16.0. Table 1 shows the two-component extraction (Rupp, in press) for the K-LoC43.

Table 1. Two Component Extraction on K-LoC43

Pattern Matrix for 2 Component Extraction on K-LoC43

	Component	
	1	2
Item13 (I)	0.685	
Item02 (I)	0.661	
Item19 (I)	0.627	
Item38 (I)	0.627	
Item21 (I)	0.601	
Item24 (I)	0.570	
Item42 (I)	0.563	
Item39 (I)	0.559	
Item10 (I)	0.558	
Item34 (I)	0.506	
Item11 (I)	0.499	
Item17 (I)	0.456	
Item03 (I)	0.414	
Item23 (I)		
Item35 (I)		
Item04 (I)		
Item12 (I)		
Item14 (I)		
Item30 (I)		
Item29 (I)		
Item43 (E)		
Item06 (E)		0.585
Item15 (E)		0.549
Item16 (E)		0.522
Item27 (E)		0.502
Item09 (E)		0.500
Item26 (E)		0.473
Item33 (E)		0.452
Item01 (E)		0.449
Item20 (E)		0.434
Item07 (E)		0.430
Item05 (E)		0.430

Item18 (E)	-0.4	0.427
Item08 (E)		0.426
Item36 (E)		0.422
Item22 (E)		0.421
Item28 (E)		
Item31 (E)		
Item37 (E)		
Item41 (E)		
Item32 (E)		
Item25 (E)		
Item40 (I)		

Extraction Method: Principal Component Analysis.
 Rotation Method: Oblimin with Kaiser Normalization.
 Rotation converged in 5 iterations.
 (E) denotes E-LoC items and (I) denotes I-LoC items according to original conception for the instrument.

For comparison, the two-component EFA on the K-LoC18 is show in Table 2. It can be seen that nearly all of the items load according to their respective constructs, whereas in the K-LoC43, numerous items failed to load.

Table 2. Two Component Pattern Matrix K-LoC18
Pattern Matrix on 2 Component Extraction K-LoC18

	Component	
	1	2
Item13 (I)	0.689	
Item11 (I)	0.684	
Item02 (I)	0.681	
Item03 (I)	0.571	
Item14 (I)	0.525	
Item10 (I)	0.513	
Item17 (I)	0.502	
Item12 (I)	0.465	
Item04 (I)	0.416	
Item06 (E)		0.659
Item15 (E)		0.658
Item05 (E)		0.557
Item09 (E)		0.549
Item16 (E)		0.515
Item01 (E)		0.513
Item07 (E)		0.453
Item08 (E)		0.411
Item18 (E)		

Extraction Method: Principal Component Analysis.
 Rotation Method: Oblimin with Kaiser Normalization.
 Rotation converged in 5 iterations.
 (E) denotes E-LoC items and (I) denotes I-LoC items according to original conception for the instrument.

Analysis of K-LoC43 based on EFA and Focus Group Outcomes

In looking at the items of the K-LoC43, it is important to note the large number of items which failed to load in a two-factor EFA extraction. This was not the case with the K-LoC18 where all items loaded at the .40 threshold, with the exception of Item 18 (Rupp, in press). Thus these non-loading items should be foremost in consideration for elimination from future revisions of the K-LoC43. The 13 items which failed to load in the two-factor EFA extraction include the following: I-LoC Items 04, 12, 14, 23, 29, 30 and 35; and E-LoC Items 28, 31, 32, 37, 41, and 43.

Beginning with the I-LoC items which failed to load, Item 04 (I decide my own life) received no comments from the students, one of only six items to be thusly ignored by the students. This may be due to the fact that it is too obvious and for students to comment upon; perhaps even laboring under a certain level of cliché or platitude. Also, as an I-LoC item, it did not engender the level of indignation, or perhaps protest, that the E-LoC items tended to. Item 12 (Usually, things turn out better if I make my own decisions) received only one comment, “[t]his is a mediocre item, because it is important to listen to others’ opinions.” This could be seen as a reflection of the traditional group-oriented nature of Japanese society (Benedict, 1967; Doi, 1973) wherein conventional members of society tend to look askance at individuals who overtly express their individuality. Item 14 (I will be able to live my entire life as I plan to) received more comments, with “I’m still only a university student” being the most sobering one among them. It is true that while many young people have high hopes for the future, especially in light of the end of the bubble economy and the troubling and competitive job market which no longer assumes a guarantee of lifetime employment, they also have anxiety about the future, and cannot necessarily buy into the idea that life is going to be easy for them. Anecdotally, many young people now complain that the future of the pension system and higher taxes due to the aging society, combined with a lack of clear support for young families, leaves them feeling with unease about their future lives in Japanese society. Item 23 (It is easy to get a good score on tests if I prepare for lessons and review afterwards) received dismissive remarks such as “[n]o kidding” and “[p]reparation, review and understanding are all different ... [i]f

you don't understand, all of it would be meaningless." These kinds of comments underscore the importance of not having items in the instrument which are expressed in a facile manner. The wording and concept should be subtler to avoid these kinds of negative reactions from the subjects. Item 29 (If a class in school is boring, it is because you are not interested in that subject) received a comment stating "[n]ot a good item because, haven't university students come to study what they wanted to study?" This makes the case for this item being targeted at high school students, although it could be argued that even university students have to take classes as a part of their major, which they might not be interested in. Item 30 (I always decide what I'm going to do) was criticized in its only comment, "[t]his is rather obvious, isn't it?" It would appear that comments which are too obvious do not fare well in the EFA factor loadings. This item could be considered a truism, as it is perhaps impossible to do something without deciding to do it, regardless of the underlying factors for that decision. The final non-loading I-LoC item was Item 35 (If necessary, I can suppress my desires at any time), which had the negative comment, "[t]his is just common sense," and which could be categorized as another of the overly obvious items.

Continuing with the E-LoC items which failed to load in the K-LoC43 two-factor EFA, Item 28 (When you take actions, it is more often the case that others have suggested them to you rather than you acting upon your own desires) received just one student comment, "I always act based on my own ideas." This comment could also be seen as categorizing the item as being too obvious or overly simplistic in its conception. This is another item which might have been better if it had been rephrased in a subtler manner. Most people, it would seem, would tend not to think of themselves as puppets in the hands of others, but rather acting from a sense of free will when they take actions. The next E-LoC item which failed to load was Item 31 (In your case, when it comes to test results, they are often influenced by your physical condition or other random events). This item was already discussed earlier in the section in conjunction with its appearing in the top 12 items voted NA. The next non-loading item was Item 32 (It is hard for me to do things as I have planned), which was another one of the rare items to receive no comments. It is hard to account for this, but it could be speculated that this item taps into a personal discipline construct rather than a LoC construct. Item 37 (My actions tend to end up going along with the flow of circumstances) received one student

comment, "[t]his item is mediocre because we should not be so insipid." This comment gives more support to the idea that phrases which could be interpreted as invoking a negative sense of person or self should be reconsidered. Item 41 (My grades depend on the teacher) received four comments from the students, "[i]t may be so, but I don't think we can ask such a question," "[t]his is common sense," "[i]f there needs to be some improvement, I think it is necessary," and "[e]ven if you say this, nothing can be done about it." These comments again show that the students tend to dislike items which are obvious or have no apparent purpose, or perhaps in this case, items which carry a taboo connotation, for example, here being the idea that grades are more less objective than society expects they should be. The final item which failed to load was Item 43 (I often find myself doing things that I don't like to do), which was discussed at the beginning of this section as it was the item with the highest number of NA votes.

With regards to the tabulated votes from the students' individual surveys in Appendix A, upon comparing the top items voted as appropriate (AP), and the top items voted most inappropriate (NA), with the EFA factor loadings in the K-LoC43 two-factor extraction from Table 1, it became evident that the items which received the highest numbers of AP votes tended to be items measuring I-LoC, and conversely, the items with the highest numbers of NA votes were those measuring E-LoC. This was regardless of the factor loadings, and many unpopular items loaded strongly, while some popular items failed to load. This may be due to the fact that the I-LoC items express positive sounding sentiments, such as: "If I work hard;" "If I try hard;" "I decide my own life;" "If I am kind;" "make my own decisions;" "try my best;" et cetera. In the case of E-LoC items, the sentiments expressed are typically those of a more negative world view, including phrases such as: "determined by fate;" "depends on the situation;" "others have suggested;" "we can't change;" "depends on luck;" "it is hard for me;" "doing things I don't like to do;" "life is like gambling;" "I often do impulsive things;" et cetera. The students tended to dislike answering such questions, even on a Likert scale where they could strongly disagree with them, perhaps because such questions were experienced as attacking their character. Numerous students also registered complaints about the premise of a number of E-LoC items, such as "I don't like luck" about Item 36, "[w]e should not be so insipid" about Item 37, "[n]ot a good item because we should know how to make efforts"

about Item 33, “I don’t like chance” about Item 31, “I hate the idea of fate deciding things” about Item 15, et cetera. Nevertheless, according to the factor loadings, the number of votes tabulated, both for AP and NA, is not correlated with the loading strength, and therefore, when considering a revision of the instrument, it appears that a priority should be placed on the factor loadings, rather than the appropriateness vote tabulations.

Recommended Modifications for K-LoC43

Based on the feedback from the students, the changes that should be made to the instrument are as follows:

- 1 The instrument needs to be shortened, preferably to five or six items per construct.
- 2 Repetitive items should be eliminated.
- 3 Unclear and age inappropriate items should be avoided
- 4 The Likert scale should be more refined by adding further points of discrimination to it, and the ordering of the scale should be reversed to conform with the what the students perceive to be the standard ordering.
- 5 The focus group suggestions for Japanese language changes should be adopted where appropriate or necessary

It is the author’s opinion, based on the evidence, that at a very minimum, given 1) the multitude of criticisms of the length of the K-LoC43 2) it having failed the CFA test for model plausibility 3) the shorter K-LoC18 two-factor EFA showed much better factor loadings for the underlying LoC constructs on an item per item basis, that the K-LoC18 be used in place of the K-LoC43. This should be until such time as a revised Kambara LoC scale which is even shorter, less redundant, and more refined in terms of Likert scale discrimination points can be tested and confirmed with a priori data from the Japanese population. The K-LoC18 also has the benefit of being more of a general LoC instrument and thus useable in the university context with no explicit item alterations required.

In terms of a future revision of the K-LoC43, a useful starting point would be to make a ten to twelve item scale using the 5 or 6 items (taking into consideration redundancy) from both I-LoC and E-LoC, which had the highest factor loadings (Table 1). Table 3 shows the top ten highest factor loading items for both constructs.

Based on the principles of avoiding redundancy, a modified K-LoC43 could be created using five items from each construct, I-LoC and E-LoC. For example, Item 13 and Item 6, while both being the highest loading items in the EFA, should not be included together as they are saying the same thing in opposite wording which, on the basis of responses

Table 3. Top Ten Items based on EFA Loadings

Top Ten Items based on EFA Loadings

Item	I-LoC Items	Item	E-LoC Items
13 I	My happiness or sadness is determined by my own efforts.	6 E	My happiness and sadness are determined by chance.
2 I	I can be a great success if I work hard.	15 E	My future is determined by fate or chance.
19 I	If I am careful about my actions, people will trust me.	16 E	What happens does not depend on my efforts.
38 I	The results are far better when I prepare for exams in advance.	27 E	Being able to maintain long friendships depends on the external situation.
21 I	If I try hard, I will be able to get the job I want.	9 E	Life is a gamble.
24 I	I can make friends with the opposite sex if I try.	26 E	Getting into my first choice university depends more on luck than ability.
42 I	If I am kind to my friends, someday they will help me.	33 E	We can't change how smart or stupid we are.
39 I	It is important to think about my actions in order to have good relationships with my friends.	1 E	It is best to just go with the flow.
10 I	It is useful (meaningful) to think about what I want to be in the future.	20 E	My ability to make good friends depends on the class or club's atmosphere.
34 I	Maintaining friendships depends on your effort.	7 E	What happens depends on the situation.

from the qualitative data, respondents do take positively. Also, such oppositely-worded items serve also to make the point of the survey too obvious. Thus, for example, a short 10-Item survey could be creating using for I-LoC, Items 13, 19, 38, 21 and 42; and for E-LoC, Items 15, 16, 27, 33, and 1. The cost in terms of time for such a survey is minimal and the low number of items makes the purpose unclear, yet it is sufficient to measure the constructs in question. Furthermore, respondents are more likely to answer authentically with a shorter survey as they will not suffer from fatigue, or even the expectation of fatigue which might develop as they are presented with 43 items to respond to. This survey should also use a 5 to 7 point Likert scale for more refinement, and may require the scale directionality to be reconsidered from positive (I agree/I think so) to negative (I disagree/I don't think so) , though this is a matter of judgement, opinion and convention, and should not matter if the scaling is clearly labeled.

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Appendices

Appendix A: Author's Translation of K-LoC43 with Focus Group Results

Kambara 43-Item LoC Scale

(Translated by author; including the Item Appropriateness Focus Group Voting Results for appropriateness to the university context – AP= Appropriate NS= Not Sure, NA = Not Appropriate)

Note: “I” refers to items measuring internal LoC and “E” for those measuring external LoC. Students taking the surveys do not see such information.

Item	I/E		AP	NS	NA
1	E	It is best to just go with the flow.	10	4	20
2	I	I can be a great success if I work hard.	5	1	20
3	I	Anyone will be able to understand me if I try my best to communicate with them.	5	1	19
4	I	I decide my own life.	6	1	10
5	E	My life is decided by fate.	7	9	14
6	E	My good fortune and misfortune are determined by chance.	6	5	16
7	E	What happens depends on the situation.	8	1	13
8	E	My friends can't understand me no matter how hard I try.	9	2	15
9	E	Life is like gambling.	4	8	21
10	I	It is useful (meaningful) to think about what I want to be in the future.	3	0	17
11	I	If I try hard, I can do anything on my own.	3	4	17
12	I	Usually, things turn out better if I make my own decisions.	8	0	20
13	I	My happiness or sadness will be determined by my own efforts.	4	0	14
14	I	I will be able to live my entire life as I plan to.	7	3	15
15	E	My future is determined by fate or chance.	4	6	12
16	E	What happens does not depend on my efforts.	5	7	18
17	I	I can be a friend with anyone if I try.	6	1	15
18	E	Your efforts and success are not really related to each other.	4	5	16
19	I	If I am careful about my actions, people will trust me.	6	2	16
20	E	My ability to make good friends depends on the class or club's atmosphere.	5	4	18
21	I	If I try hard, I will be able to get the job I want.	6	1	14
22	E	Marrying an ideal partner depends on fate or luck.	7	4	18
23	I	It is easy to get a good score on tests if I prepare for lessons and review afterwards.	7	1	16

Recommended Modifications to the Kambara Locus of Control Scale

24	I	I can make friends with the opposite sex if I try.	6	2	14
25	E	I often do impulsive things without being aware of them.	9	2	11
26	E	Getting into my first choice university depends more on luck than ability.	7	7	17
27	E	Being able to maintain long friendships depends on the external situation (environment?) .	6	1	19
28	E	When you take actions, it is more often the case that others have suggested them rather than you acting upon your own desires.	5	1	17
29	I	If a class in school is boring, it is because you are not interested in that subject.	5	2	20
30	I	I always decide what I'm going to do.	4	0	13
31	E	In your case, when it comes to test results, they are often influenced by your physical condition or other random events.	6	6	18
32	E	It is hard for me to do things as I have planned.	5	1	15
33	E	We can't change how smart or stupid we are.	5	5	19
34	I	Maintaining friendships depends on your effort.	5	0	16
35	I	If necessary, I can suppress my desires at any time.	7	1	10
36	E	There is no use in thinking about how to make friends with members of the opposite sex as such things are determined by fate/depend upon luck.	7	7	17
37	E	My actions tend to end up going along with the flow of circumstances.	6	1	21
38	I	The results are far better when I prepare for exams in advance.	3	0	20
39	I	It is important to think about my actions in order to have good relationships with my friends.	4	1	17
40	I	Even if my friends have different ideas, I place a priority on my own actions.	7	1	18
41	E	My grades depend on the teacher.	3	3	18
42	I	If I am kind to my friends, someday they will help me.	6	2	0
43	E	I often find myself doing things that I don't like to do.	2	17	6

Appendix B: General Focus Group Feedback on K-LoC43

Student	Author's Translation of Written Student Comments
1	Some of the Japanese seems a bit unnatural. It was difficult as these are questions that we usually never think about.
2	The contents of many of these questions are a bit hard for people around 20 years old. For number 1, maybe adding "I think.." to it would be better.
3	Overall, there are many questions that are hard to answer. The Japanese used in the questions is sometimes unnatural, so I would fix those areas.
4	Overall the content is rather deep. There were many questions that I didn't understand the intentions of.
5	There were many questions which didn't take into account various degrees or levels or gradations of answer. Also, there were a few questions for which it was hard to understand what was really being asked.
6	I felt like these questions were trying to find out what kind of a human being I am!
7	It seems like a lot of the questions are similar, merely asking the same thing in the opposite fashion.
8	There were a lot of hard items. Numbers 24 and 36 seemed the same. There were many questions that seemed the same.
9	I can't figure out what these questions have to do with English class. There were many questions that seemed similar.
10	(no comment)
11	This was difficult.
12	This was hard. It seemed like philosophy. There is no answer even after thinking for a long time. Many questions were similar. For example, you could just have Item 4 or Item 5, and that would be sufficient.
13	The questions about "life" were really hard to answer. Some of the questions were good for self-reflection.
14	These made me think about life and my actions.

15	I think you could see a person's viewpoints on life through this questionnaire. Basically it seems to be asking "effort" or "luck"?
16	There were some hard questions. I couldn't figure out what the aim of this questionnaire was.
17	There were a number of questions that we don't see on typical questionnaires, so it was kind of interesting. I would love to see the results of this questionnaire. It was unusual to see questions using the words "luck" and "fate".
18	People act on their own volition, so I think the answers are going to depend on the person.
19	(no comment)
20	I was surprised by this questionnaire because I was expecting something related to our classwork. However, I thought it was meaningful.
21	At the end of the day, I think I decide my own outcomes.
22	I felt like the contents weren't fully fleshed out, the questions were too abrupt and this could cause misunderstandings. It seems like there were a lot of questions that were more like "digs" at us. LOL
23	I don't get this questionnaire.
24	I don't understand the point of this questionnaire.
25	I was made to rethink how I live my life. Many questions were very similar, gradually it became like leaving it up to luck.
26	There is no need to make friends with members of the opposite sex. I think answering all these questions from 1 to 4 is not as good as expressing your opinions about these items.
27	There are a lot of similar questions and negative questions. Some of the questions were helpful, though.

Appendix C: Author's Translation of Student Comments on each item of K-LoC43

Item 1 It is best to just go with the flow.

- * This resembles question 9.
- * This is not good because I am at an age where my choices are respected.
- * I don't think everything would necessarily go well.
- * I think you could get the wrong idea with this one and end up not making any effort.

Item 2 I can be a great success if I work hard.

- * I think the word "great success" is a bit abstract.

Item 3 Anyone will be able to understand me if I try my best to communicate with them.

- * This is too idealistic.

Item 4 I decide my own life.

(No Comments)

Item 5 My life is decided by fate.

- * I have only lived for 20 years.
- * University student life depends upon hard work
- * I have only live for 20 years.
- * This is not a good question. It seems like it is asking the same thing as number 4.
- * People can change their lives.
- * This is similar to item 4.
- * I don't want this to be true.
- * It is not only fate but also your destiny. Both of these decide your life.

Item 6 My happiness and sadness are determined by chance.

- * This is a heavy topic for university students.
- * I still don't know.
- * I don't want this to be true.
- * I think this depends on my efforts.
- * I feel that the word "chance" lacks a sense of responsibility.

Item 7 What happens depends on the situation.

- * This is a heavy topic for university students.
- * A questionable item as it ends up making me look like I am disregarding my own person.
- * What happens around you depends on your attitude.

Item 8 My friends can't understand me no matter how hard I try.

- * Not a good item because university students should be able to make themselves understood, at least to some extent.

Item 9 Life is a gamble.

- * This resembles question 1
- * This really depends on the person.
- * This is the same as no. 5. Also, I'm pretty sure there aren't any university students who would say something this outrageous.
- * I don't like the idea of things being decided by fate.
- * I don't understand this question.

Item 10 It is useful (meaningful) to think about what I want to be in the future.

- * Depending on the person it might turn out bad.

Item 11 If I try hard, I can do anything on my own.

- * If you try your best things may be only easier to do.
- * The people around you are definitely going to be involved.

Item 12 Usually, things turn out better if I make my own decisions.

- * This is a mediocre item, because it is important to listen to others' opinions.

Item 13 My happiness or sadness is determined by my own efforts.

- * Happiness depends on how you view things.

Item 14 I will be able to live my entire life as I plan to.

- * I'm still only a university student.
- * I plan my own life.
- * Does this mean will I be able to overcome obstacles?
- * I hate the idea of fate deciding things.

Item 15 My future is determined by fate or chance.

- * I don't think you can say everything is up to chance or fate.
- * I'm still only a university student.
- * I hate the idea of fate deciding things.
- * It is not only chance but also effort combined with it, as a minimum requirement.

Item 16 What happens does not depend on my efforts.

- * This might be better asked of adults.
- * I don't think this is how things work.
- * Not a good item, because we have to make great efforts in order to find a job.
- * This question seems like "giving up on it all".
- * You can do something on your own. This question is not appropriate for university students.

Item 17 I can be friends with anyone if I try.

(No Comments)

Item 18 Your efforts and success are not really related to each other.

- * I don't think this is how things work.
- * This depends on the person.

Item 19 If I am careful about my actions, people will trust me.

- * This item is mediocre because we should be careful about our own actions in any event.
- * If you are careful about your words and actions and take consideration to your situation, at some point people are going to have to trust you.
- * There can sometimes be problems with personality/character.

Item 20 My ability to make good friends depends on the class or club's atmosphere.

- * Not a good item because we should be able to make friends regardless of the atmosphere.

- * In university, unlike up until high school, it depends on the person.

Item 21 **If I try hard, I will be able to get the job I want.**

- * Sometimes there are job "ice ages".

Item 22 **Marrying an ideal partner depends on fate or luck.**

- * I can't imagine.
- * I really don't know, as I am still a college student.
- * I think you should ask someone around 30 years old.
- * University students are rarely married, so this is hard to think about.
- * Ideals and reality can be different, if you try hard, you might get lucky.

Item 23 **It is easy to get a good score on tests if I prepare for lessons and review afterwards.**

- * Preparation, review and understanding are all different. If you don't understand, all of it would be meaningless.
- * No kidding!

Item 24 **I can make friends with the opposite sex if I try.**

- * We don't need this question about "opposite sex".

Item 25 **I often do impulsive things without being aware of it.**

- * I don't really get what this question is asking.
- * I'm not sure what the aim of this question is.

Item 26 **Getting into my first choice university depends more on luck than ability.**

- * Maybe you should change this to getting into "a company".
- * This is a question for high school students.
- * I hate things like "chance".
- * You get into university based on your ability.
- * For those students who are attending a university that wasn't their first choice, this is a tough question.
- * Getting into the first choice university requires both effort and luck.
- * We have already entered university, so this is unnecessary.

Item 27 **Being able to maintain long friendships depends on the external situation (environment).**

- * Not a good item because university students should be able to maintain friendships regardless of the situation.
- * Personal relationships are getting weaker these days, so it depends on the person.

Item 28 **When you take actions, it is more often the case that others have suggested them rather than you acting upon your own desires.**

- * I always act based on my own ideas.

Item 29 **If a class in school is boring, it is because you are not interested in that subject.**

- * Not a good item because, haven't university students come to study what they wanted to study?

Item 30 **I always decide what I'm going to do.**

- * This is rather obvious, isn't it?

Item 31 **In your case, when it comes to test results, they are often influenced by your physical condition or other random events.**

- * This is just an excuse, so I don't think there is any need for this question.
- * Not good, I'm pretty sure we can't use our physical condition as an excuse (LOL).
- * I don't like "chance".
- * This is just an excuse.
- * Are we talking about children here?

Item 32 **It is hard for me to do things as I have planned.**

(No Comments)

Item 33 **We can't change how smart or stupid we are.**

- * Not a good item because we should know how to make efforts!
- * One's intelligence or lack thereof is based on effort.
- * Someone's efforts can have an effect.
- * I think you get smarter and smarter by working hard.

Item 34 **Maintaining friendships depends on your effort.**

(No Comments)

Item 35 **If necessary, I can suppress my desires at any time.**

- * This is just common sense.

Item 36 **There is no use in thinking about how to make friends with members of the opposite sex as such things are determined by fate/depend upon luck.**

- * Not good because we should be positive about any aspects of our lives.
- * I don't like "luck".

Item 37 **My actions tend to end up going along with the flow of circumstances.**

- * This item is mediocre because we should not be so insipid.

Item 38 **The results are far better when I prepare for exams in advance.**

(No Comments)

Item 39 **It is important to think about my actions in order to have good relationships with my friends.**

- * This is common sense.

Item 40 **Even if my friends have different ideas, I place a priority on my own actions.**

- * This is common sense.
- * This is a mediocre item because university students should not be so self-centered. LOL.

Item 41 **My grades depend on the teacher.**

- * It may be so, but I don't think we can ask such a question.
- * This is common sense.
- * If there needs to be some improvement, I think it is necessary.
- * Even if you say this, nothing can be done about it.

Item 42 **If I am kind to my friends, someday they will help me.**

- * I have a problem with the phrasing "If I am kind".
- * This is common sense.
- * This is a mediocre item because I don't think I would try to make friends with the idea of someday being helped by them.

Item 43 **I often find myself doing things that I don't like to do.**

(No Comments)

要旨

本論文は英語教育などにおける学習者の自律 (learner autonomy: LA) と密接にかかわるデータ抽出手法である、鎌原 (1982, 1987) が開発したアンケート「統制の所在: LoC」(The Kambara Locus of Control Scale (K-LoC) (18項目版および43項目 (拡大) 版) によって得られたデータを、心理測定学的 (psychometric) 観点から実証的に解析・検証したものである。研究にあたって、1125名の高校生からデータを収集し、得られたデータを確認的因子分析 (CFA) および探索的因子分析 (EFA) により解析している。併せて、定性的研究手法である「フォーカス・グループ」を用いて、27名の大学生からグループディスカッション形式の発話データなどを収集し、計量的解析に質的解析を加えることで検証の妥当性を高めることを試みている。量的のデータ分析に加えて質的のデータ分析で K-LoC への改善や変更を目指している。K-LoCS への修正提案や「フォーカス・グループ」法によって収集したデータから明らかになったことを念頭に、本論文のまとめが記述されている。

ヤーコン茶葉熱水抽出物のリポキシゲナーゼに対する阻害作用および RAW264.7マウス由来マクロファージ様細胞を用いた NO 産生抑制作用

上田裕人*・菅原進太郎*・松田 靖***・村田達郎***・黒田泰弘***・星 良和***
梶田聖孝***・小野政輝***・井越敬司***・安田 伸***

Effects of Hot-water Extract of Herbal Tea Leaves from Yacon (*Smallanthus sonchifolius*): Lipoygenase Inhibition and Suppression on Nitric Oxide Generation in RAW264.7 Mouse Macrophage-like Cells

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ABSTRACT

Yacon (*Smallanthus sonchifolius*), has been believed as a potent alternative food source for patients who require a dietary cure in regional area. It has been used as a folk medicine for the people suffering from diabetes and digestive/renal disorders. The leaf part of this plant is locally consumed as herbal tea, and recognized as a health food material due to the rich in polyphenol. However, the mechanisms underlying the functional properties of this plant and even its processed foods remain to be completely established. We have previously evaluated antioxidant capacity of herbal tea leaves from yacon. Therefore, it is important to evaluate the multi-functionality of this herbal tea leaves. This study aims to examine anti-inflammatory effects of hot-water extract of herbal tea leaves from yacon. In lipoygenase (LOX) inhibition assay, the hot-water extract resulted in demonstrating a concentration-dependent inhibition, with IC₅₀ value of 39.5 µg/ml. Subsequently, macrophages cultured in the presence of hot-water extract showed suppressing effect on nitric oxide (NO) production in a lipopolysaccharide-activated cell model, with IC₅₀ value of 35.4 µg/ml under the non-toxic concentration. In addition, the hot-water extract showed weak NO scavenging activity. These results may indicate the potential of the herbal tea leaves from yacon to manage LOX and NO-mediated inflammation.

緒 言

ヤーコン (*Smallanthus sonchifolius*) はキク科スマランサス属の一種であり、南米アンデス山脈地方原産の多年生草木である。過去数十年に渡り、ヤーコンはアジア (日本、韓国、台湾、海南およびフィリピン)、オセアニア (ニュージーランド)、そしてヨーロッパ (チェコ共和国) などの様々な場所に導入されてきた (1)。この植物の塊根は、サツマイモのような形状であり、歴史的に果物/野菜として摂取されており、現在では、南米にてシロップ、ジュース、マーマレードなどの加工食品とし

て利用されている (1,2)。最近では、ヤーコンは地域によっては食事療法を必要とする患者のための潜在的な代替食として考えられている (2)。また、糖尿病や消化/腎障害を患う人々のための民間薬としても使用されている。これは豊富な難消化性フラクトオリゴ糖と有益なポリフェノールに起因する可能性が示唆されている (3)。さらにヤーコン塊根は、抗酸化作用 (4)、ラットにおける抗糖尿病作用 (5)、ヒトにおける抗肥満 (6) および糖尿病ラットに対する脂質低下作用 (7) が研究されている。ヤーコン葉部の一部はハーブティーとして地域限定ながらも市販化ならびに消費されているものの、そのほとんどは食材として利用されておらず、有効利用の開発が必要である。また葉部には、豊富なフェノール性化合物が含有されている。その主成分としてクロロゲン酸、カフェ酸、フェルラ酸等が挙げられ (8)、さらにはヤーコン葉特有のカフェオイル誘導体が含有されていること

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(9) が報告されている。またヤーコン葉部は、抗真菌作用 (10)、抗酸化作用 (11)、正常および糖尿病ラットモデルにおける血糖降下作用 (12) を有することが報告されている。これまでに我々は、ヤーコン茶葉の機能性研究に着手しており、これまでに複数のフリーラジカル種に対する抗酸化作用、特に活性酸素種の 1 つであるスーパーオキシドアニオン (O_2^-) ラジカルに対する抑制作用について酸化酵素やヒト顆粒球様好中球細胞モデルを用いて明らかにしてきている (13)。我々は、本植物ならびにその加工食品の新たな機能性として、抗炎症作用に着目している。

炎症は、病原体、化学または物理的損傷の存在に対するヒトおよび他の哺乳動物の応答反応である。炎症反応には、いくつかの起炎酵素が炎症性メディエーターの産生や組織損傷の亢進に寄与することが知られている。リポキシゲナーゼ (LOX) は、喘息、乾癬、癌転移およびアテローム性動脈硬化症のような多くの病態生理学的プロセスにおいて、炎症性ロイコトリエン、リポキシンおよびヒドロキシエイコサテトラエン酸を生成する重要な酵素である (15,16)。また、LOX が急性皮膚炎 (17) や *in vivo* でのヒスタミン誘発性浸潤 (18) に関与していることが報告されている。このように様々な炎症関連プロセスに本酵素が寄与していることから、アラキドン酸カスケードにおける LOX 経路を天然物が阻害しうるかどうかを調べることに関心が寄せられている (19,20)。血液中の単球に由来するマクロファージ細胞は生体防御の上で免疫反応に重要な役割を担う。サイトカインや微生物性の物質はマクロファージを活性化し、そこでは過剰量の一酸化窒素 (NO) が産生されうるため、NO レベルは *in vitro* および *in vivo* でのマクロファージの炎症状態を反映する (21,22)。また、過度の NO 産生によって敗血症、関節リウマチの他に出血性ショック、全身性エリテマトーデス、シェーグレン症候群、血管炎、変形性関節炎、乾癬、接触性皮膚炎のような炎症性疾患の進行が生じることが指摘されている (23)。我々はこれまでに、薬用植物であるツルボ (*Scilla scilloides* DRUCE (Liliaceae)) や地域の特徴ある食材としての食用イグサ (*Juncus effusus* L. (Juncaceae)) の有する抗炎症作用について研究を行い、両者が起炎酵素である LOX を阻害すること、マクロファージ様の細胞モデルにおいて NO 産生抑制作用を示すことを報告してきた (24,25)。

本研究では、ヤーコン茶葉熱水抽出物の潜在的な抗炎症作用について調べるため、*in vitro* で LOX に対する阻害作用およびマクロファージ様細胞モデルにおける NO 産生抑制作用について調べることにした。

材料および方法

原材料および試薬類

‘サラダオトメ (旧系統名: SY201)’ は、日本で品種登録されているヤーコンの一つである (26)。本研究で用いた乾燥ヤーコン茶葉は、本品種を菊池市 (熊本県) で栽培、茶葉への加工およびハーブティーとして販売されているものを 2011 年 3 月に入手したものである。ノルジヒドログアイアレン酸 (NDGA) は MP Biomedicals LLC (米国) より、メチレンブルー、リン酸二水素ナトリウム、リン酸水素二ナトリウム、リノール酸、ジメチルスルホキシド (DMSO)、ペニシリン G、ストレプトマイシン、*N*-1-ナフチルエチレンジアミン二塩酸塩、水酸化ナトリウム、塩化ナトリウム、塩化カリウムはナカライテスク (京都) より入手した。DMEM 培地、ポリオキシエチレン (20) ソルビタンモノラウレート (Tween20)、リン酸は和光純薬工業株式会社 (大阪) より、ペンタシアノニトロシル鉄 (III) 酸ナトリウム二水和物 (SNP)、(±)-6-ヒドロキシ-2,5,7,8-テトラ-メチルクロマン-2-カルボン酸 (trolox)、ダイズ (*Glycine Max*) 由来リポキシゲナーゼ、大腸菌 (*Escherichia coli*) 由来リポ多糖 (LPS)、スルファニルアミドはシグマアルドリッチ社 (米国) より購入した。Cell Counting Kit-8 (WST-8) は同仁化学研究所 (熊本) より、クルクミンはキシダ化学株式会社 (大阪) より、RAW264.7 マウス由来マクロファージ様細胞 (ECACC91062702) は DS ファーマバイオメディカル株式会社 (大阪) より購入した。ウシ胎児血清 (FBS) はギブコ、インビトロジエン (米国) より購入した。Griess 溶液は、1% スルファニルアミド、0.1% *N*-1-ナフチルエチレンジアミン二塩酸塩および 2.5% リン酸を含む溶液から成る。10 mM リノール酸溶液は、リノール酸 56 mg、Tween20 112 mg、MilliQ 35.2 μ l を転倒混和し、1 M 水酸化ナトリウム 24 μ l、MilliQ 776 μ l を含む溶液から成る。

ヤーコン茶葉熱水抽出物の調製

ヤーコン茶葉熱水抽出物は、以前の報告で調製されたものを使用した (11)。簡潔に述べると、粉碎した茶葉 5 g をコンカルビーカーに入れ、90~100°C の MilliQ 水 250 ml 中で 45 分間浸漬した。茶葉の懸濁液を遠心分離し、濾過した。その後、濾液を凍結乾燥させ、水で再溶解させたものを以降の実験に使用した。

リポキシゲナーゼ (LOX) 阻害活性測定

LOX 阻害活性は、酵素反応中にリノール酸の酸化に

伴うメチレンブルーの退色を指標に測定した (24)。これは既報の異なる LOX 活性測定技術 (27,28) を我々が改良したものである。96ウェルプレートに試料溶液を 17.5 μ l, 0.2 M リン酸緩衝液 (pH 6.0) を 140 μ l を混合させ、さらに MilliQ を 102.9 μ l, 5 mM リノール酸溶液を 5.6 μ l, 250 μ M メチレンブルーを 14 μ l, 212 kU/ml LOX を 70 μ l それぞれ混合した。マイクロプレートリーダー (SH-1000Lab, コロナ電気, 茨城) を用いて 660 nm における吸光度を測定した。その後、遮光下で 30 分間反応後同様に測定を行なった。ポジティブコントロールには NDGA を使用した。

阻害活性 (%) は、次式により算出した。

$$\text{Inhibition Activity}(\%) = [1 - (A_{\text{Sample}}) / A_{\text{Control}}] \times 100$$

A_{Sample} : 試料の 30 分反応後の吸光度より 0 分の吸光度を差し引いた値

A_{Control} : 試料を含まないコントロールの 30 分反応後の吸光度より 0 分の吸光度を差し引いた値

RAW264.7 マウス由来マクロファージ様細胞の培養法

RAW264.7 マウス由来マクロファージ様細胞は、10% FBS および 1% ペニシリン-ストレプトマイシンを含む DMEM 培地を用いて、37°C, 5% CO₂ の条件下で培養した。

WST-8 法による細胞生存率の測定

細胞生存率の測定は、WST-8 法を用いて行った。即ち、RAW264.7 細胞を 50×10^4 cells/ml となるように調整し、96ウェルプレートに 100 μ l ずつ播種した。3 時間前培養した後、試料溶液を 25 μ l ずつ添加し、さらに 24 時間培養した。培養後、リン酸緩衝生理食塩水 (PBS(-) (Phosphate buffered saline, Mg²⁺, Ca²⁺-free)) で希釈された WST-8 溶液を 20 μ l 添加し、さらに 1.5 時間培養を行なった。その後、マイクロプレートリーダーを用いて 450 nm における吸光度を測定した。

細胞生存率 (%) は、次式により算出した。

$$\text{Relative Cell Number} = (A_{\text{Sample}} - A_{\text{Blank}}) / (A_{\text{Control}} - A_{\text{Blank}})$$

A_{Sample} : 試料処理後の吸光度

A_{Control} : 試料を含まないコントロールの吸光度

A_{Blank} : ブランクの吸光度

活性型 RAW264.7 細胞の NO 産生抑制効果の測定

RAW264.7 細胞を 50×10^4 cells/ml となるように調整し、96ウェルプレートにそれぞれ 100 μ l ずつ播種した。3 時間前培養を行なった後、試料溶液または終濃度 100 ng/ml になるように調製した LPS を含む培地を合計 25 μ l 添

加し、さらに 24 時間培養した。培養後上清中に放出された NO 量の測定は Marcocci ら (29) の Griess 法により測定した。即ち、培養後に得られた培養上清 100 μ l と Griess 溶液 100 μ l を混合させ、マイクロプレートリーダーを用いて 550 nm における吸光度を測定した。

このとき 100 ng/ml LPS で処理した際に認められた NO 産生量を 100% とし、サンプル添加時の NO 産生率 (%) を以下の式により算出した。

$$\text{NO Production}(\%) = (A_{\text{Sample}} - A_{\text{Blank}}) / (A_{\text{LPS}} - A_{\text{Blank}}) \times 100$$

A_{Sample} : LPS 刺激時の試料処理後の吸光度

A_{LPS} : LPS 刺激のみの吸光度

A_{Blank} : ブランクの吸光度

NO 消去活性の測定

NO 消去活性は、SNP を用いて PBS(-) 中にて自発的に生じる NO 量を Griess 試薬を使用し測定する Marcocci ら (29) の方法に基づいて行なった。1.5 ml マイクロチューブに試料溶液を 5 μ l, PBS を 45 μ l, 10 mM SNP 溶液を 50 μ l とを混合させ、室温で 150 分間静置した。その後、Griess 試薬 100 μ l により発色させ、マイクロプレートリーダーを用いて 550 nm における吸光度を測定した。ポジティブコントロールには trolox およびクルクミンを使用した。

NO 消去活性は次式で求め、消去率 (%) で表した。

$$\text{NO Scavenging Activity}(\%) = [1 - (A_{\text{Sample}} - A_{\text{Blank}})] / (A_{\text{Control}} - A_{\text{Blank}}) \times 100$$

A_{Sample} : 試料の吸光度

A_{Control} : 試料を含まないコントロールの吸光度

A_{Blank} : ブランクの吸光度

統計解析

実験結果は 4 つの異なるデータより得た平均値 \pm 標準偏差 (mean \pm S.D.) で表記した。また統計的な差は学生 t 検定を用いて $P < 0.001$ で有意であるとみなした。

結果および考察

ヤーコンは主に塊根部分が食用とされる植物であるが、茎葉部はハーブティーとして利用されている。近年では、ヤーコンは地域によっては食事療法を必要とする患者のための潜在的な代替食として考えられている (2)。我々は、本植物ならびにその加工食品の新たな機能性として、抗炎症作用に着目している。これまでに我々は、ヤーコン茶葉が複数のフリーラジカル種に対して抗酸化作用を

示すこと、特に活性酸素種の1つである O_2^- ラジカルに対する抑制作用について明らかにしてきた (13)。近年、健康志向食品素材として注目されつつあるヤーコンの健康維持および増進に有益な情報を提供するべく、本研究では、ヤーコン茶葉の熱水抽出物を用いて、起炎酵素である LOX に対する阻害活性およびマクロファージ様細胞モデルにおける NO 産生抑制作用を調べることとし、潜在的な抗炎症作用について検証を行うこととした。

ヤーコン茶葉の LOX 阻害活性

最初に、ヤーコン茶葉熱水抽出物が LOX 酵素活性を阻害するか検討した。その結果、ヤーコン茶葉熱水抽出物は濃度依存的な阻害活性の上昇を示した (Fig. 1)。ヤーコン茶葉熱水抽出物の半数阻害濃度 (IC_{50}) 値は $39.5 \mu\text{g/ml}$ であった。ポジティブコントロールとして用いた NDGA の IC_{50} 値は $3.67 \mu\text{g/ml}$ であった。我々はこれまでに、薬用植物であるツルボ、食用イグサおよび抹茶の LOX 阻害活性について調べてきた。そこでは、ツルボ酢酸エチル抽出物の IC_{50} 値には $31.5 \mu\text{g/ml}$ (24)、食用イグサの熱水抽出物およびエタノール抽出物にはそれぞれ $123 \mu\text{g/ml}$ および $143 \mu\text{g/ml}$ (25)、さらに抹茶の熱水抽出物およびエタノール抽出物にはそれぞれ $138 \mu\text{g/ml}$ および $145 \mu\text{g/ml}$ の値を得ており (25)、それぞれに異なるレベルで LOX 阻害作用を認めている。本研究で用いた抽出物は、我々が以前報告した際に調製したものであり、熱水抽出物 1 mg 当たり 0.279 mg のポリフェノールが含まれていること、収率より乾燥茶葉 1 g 当たり 0.0776 g のポリフェノールが含まれていることが明らかとなっている (13)。また、これまでに植物体に含有されるフェノール性化合物によっては、LOX 酵素阻害を示すものがあることが報告されており (30-32)、緑茶由来カテキン類の摂取は、LOX 阻害を介して *in vivo* での急性炎症を抑制することが報告されている (33)。ヤーコン茶葉のフェノール性の主成分にはクロロゲン酸、カフェ酸、フェルラ酸等が挙げられ (8)、これらが成分レベルで LOX 阻害作用を示すことが報告されている (34,35)。現時点ではヤーコン茶葉熱水抽出物に含まれる LOX 阻害に関する成分レベルでの知見は未だ得られていない。ヤーコン葉特有のカフェオイル誘導體 (9) が特に強い LOX 阻害作用を示すことが報告されており (36)、これらに類するポリフェノール成分が LOX 阻害に対して大きく貢献している可能性がある。従って、ヤーコン茶葉熱水抽出物は、食用イグサや抹茶の熱水抽出物あるいはエタノール抽出物よりも LOX に対して強い阻害作用を有し、薬用植物のツルボの酢酸エチル抽出物に匹敵する

ほどの阻害作用を有することが認められた。

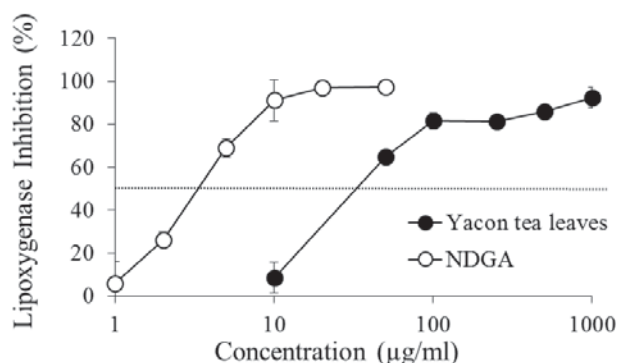


Fig. 1. Effect of concentration of hot-water extract from yacon tea leaves in lipoxigenase inhibition assay. Data shown represent mean \pm S.D. from four different experiments. NDGA was used as the standard sample. NDGA; nordihydroguaiaretic acid.

ヤーコン茶葉のマクロファージ細胞モデルにおける NO 産生抑制

次に、ヤーコン茶葉熱水抽出物が LPS で刺激した活性型マクロファージ様細胞の過剰な NO 産生を抑制するかについて調べた。まず初めに細胞毒性試験を行うべく、細胞をサンプルとともに24時間培養し、WST-8法を用いて比色定量により生細胞数を求め、これを細胞生存率の指標とした。その結果、未処理の生細胞数を100%としたとき、 $10\sim 200 \mu\text{g/ml}$ の濃度範囲でヤーコン茶葉熱水抽出物は、有意な生細胞数の低下を示さず、細胞生存率に影響を及ぼさなかった (Fig. 2A)。これにより、 $200 \mu\text{g/ml}$ までの非毒性濃度範囲でマクロファージ細胞モデルにおける NO 産生抑制作用を調べることとした。RAW264.7細胞を 100 ng/ml の LPS により刺激し、これを炎症時の活性型マクロファージのモデルとした。ヤーコン茶葉熱水抽出物を LPS とともに24時間処理し、上清中の NO 産生量に及ぼす影響を調べた。その結果、LPS 誘導の NO 産生量を100%としたとき、ヤーコン茶葉熱水抽出物は $10\sim 200 \mu\text{g/ml}$ の濃度範囲において濃度依存的に有意な NO 産生抑制作用を示した ($P < 0.001$)。算出された IC_{50} 値は $35.4 \mu\text{g/ml}$ であった (Fig. 2B)。これまでに異なる8種類のハーブティー (デージーティー、ジャスミンティー、ラベンダーティー、ローズティー、モクセイティー、レモングラスティー、ローズマリーティー、緑茶) のマクロファージ様細胞における NO 産生抑制作用について報告があり、その中では $113 \mu\text{g/ml}$ の IC_{50} 値とともに緑茶が最も強い抑制作用を有している (37)。また我々は、食用イグサおよび抹茶の RAW264.7細胞を用いた NO 産生抑制作用についても調べており、

食用イグサの熱水抽出物およびエタノール抽出物の IC₅₀ 値はそれぞれ 120 μg/ml および 35.2 μg/ml, ならびに抹茶の熱水抽出物およびエタノール抽出物の IC₅₀ 値はそれぞれ > 250 μg/ml および > 100 μg/ml であることを報告している (25). ヤーコン茶葉熱水抽出物で得られた本結果は, 他の報告例と比べてもより強力なものであった. ヤーコン葉は TNF- α , PGE₂ といったエイコサノイド類の産生に対しても阻害作用を有しており, セスキテルペンラクトン類が局所抗炎症作用に寄与している (38). 現時点ではヤーコン茶葉中に含まれるマクロファージモデル細胞の NO 産生抑制成分に関する知見は得られていないものの, 本研究で用いたヤーコン茶葉熱水抽出物は, 細胞レベルで強い NO 産生抑制作用を示したことより, NO が引き起こす炎症性疾患のリスクを軽減させる可能性が考えられた.

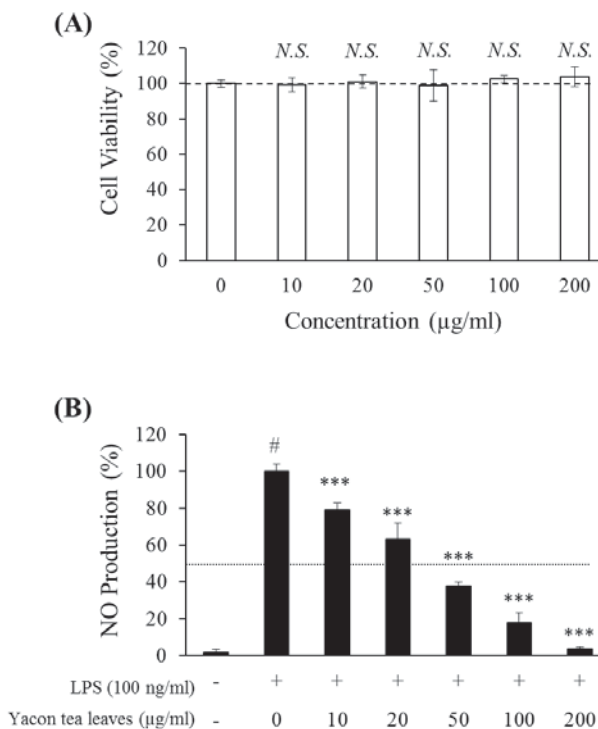


Fig. 2. Effect of hot-water extract from yacon tea leaves on cell viability (A), and LPS-stimulated NO production in RAW264.7 mouse macrophage-like cells (B). In (A), cells were incubated in the presence of varying concentrations of the extract for 24 h. Cell viability was measured using Cell-counting kit-8. In (B), cells were incubated in the presence of the extract plus 100 ng/ml LPS for 24 h. The culture media were collected and subjected to determination of NO levels by the Griess method. Data shown represent mean \pm S.D. of four different experiments. [#]Significant difference from untreated cells (control) was considered at $P < 0.001$, and ^{***}significant difference from LPS-treated cells, $P < 0.001$. N.S. indicates no significance from control in (A). NO; nitric oxide, LPS; lipopolysaccharide.

ヤーコン茶葉熱水抽出物が RAW264.7細胞の NO 産生を抑制することが確認されたものの, これらの結果は, 細胞中の NO 合成経路を直接的または間接的に抑制することによるのか, または細胞より放出された NO 自体を特異的に消去したためか疑問が残る. そこで PBS 中に NO ドナーである SNP を入れることにより, 疑似的に溶液中に NO を生成させ, その NO をヤーコン茶葉熱水抽出物が消去しうるかについて調べた. その結果, 濃度依存的な消去活性の上昇が認められ, 738 μg/ml の EC₅₀ 値が得られた (Fig. 3). しかしながら, 前述の細胞実験に使用した 10-200 μg/ml の濃度範囲では約 8-45% の消去率を示すのみであったことより, ヤーコン茶葉熱水抽出物は弱いながらも高濃度では NO を直接的に消去しうること, 200 μg/ml 以下の低濃度領域では直接的または間接的に細胞に作用し, NO 合成経路に干渉して細胞の NO 産生を抑制する可能性が考えられた. この時のポジティブコントロールとして用いたクルクミンおよび trolox の EC₅₀ 値はそれぞれ 18.0 μg/ml および 733 μg/ml で

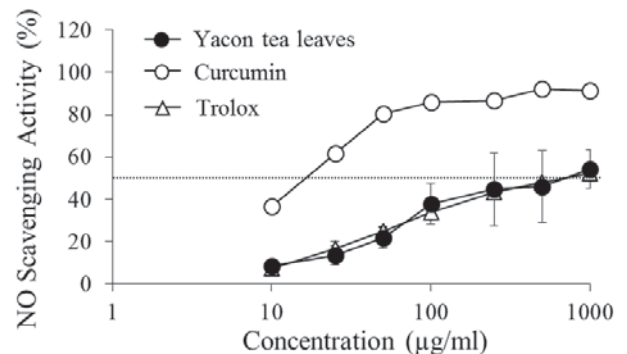


Fig. 3. Effect of concentration of hot-water extract from yacon tea leaves in NO scavenging assay. Data shown represent mean \pm S.D. from four different experiments. Curcumin and trolox were used as the standard sample. NO; nitric oxide.

あった.

ヤーコン茶葉の機能特性に関する科学的エビデンスの付与を目的に, ヤーコン茶葉熱水抽出物を用いて *in vitro* での抗炎症作用を評価した. LOX に対して濃度依存的な阻害作用を有し, 既報の薬用植物と同等の阻害能を持つことを見出した. また, LPS 刺激した活性型 RAW264.7マウス由来マクロファージ様細胞より過剰産生される NO に対して濃度依存的かつ有意な阻害能を持つこと, 作用メカニズムは NO に対して直接消去するよりも, マクロファージ様細胞内の NO 合成経路に直接的または間接的に干渉して, 抑制的に作用する可能性が考えられた. 本研究で用いたヤーコン茶葉熱水抽出物は,

O₂⁻ラジカルを中心に多彩な抗酸化能を有することを我々は報告しており (13), 多機能性を有する高機能性食品として, 本加工食品の有効活用が期待される. しかしながら, ヤーコン茶葉が有する抗炎症作用の解明には, ヤーコン茶葉の NO 産生抑制メカニズムの解明, ヤーコン茶葉由来の有効成分の分離同定や定量, 分子レベルでの評価, 生体レベルでの評価など, 更なる検証が必要である.

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要 約

ヤーコン (*Smallanthus sonchifolius*) は, 地域によっては食事療法を必要とする患者のための潜在的な代替食として考えられ, 糖尿病や消化/腎障害を患う人々のための民間薬としても使用されている. 一方, ヤーコン葉部の一部はハーブティー等として地域限定ながら流通しており, 豊富なポリフェノールを含む健康食品素材として注目される. 我々は, 本植物ならびにその加工食品の新たな機能性として, 抗炎症作用に着目している. これまでに我々は, ヤーコン茶葉の抗酸化作用について評価を行ってきた. ヤーコン茶葉の持つ多角的な機能性を評価することは重要であり, 抗炎症作用の観点よりヤーコン茶葉の機能性について調査を開始することとした. ヤーコン茶葉熱水抽出物が, 起炎酵素であるリポキシゲナーゼに対して阻害作用を有するかを調べた結果, 39.5 µg/ml の IC₅₀値とともに濃度依存的な阻害作用が認められた. 次に RAW264.7マウスマクロファージ様細胞の NO 産生に及ぼす影響を調べた結果, 毒性を示さない濃度で 35.4 µg/ml の IC₅₀値とともに濃度依存的な抑制作用が認められた. 一方, 本熱水抽出物は, それほど強い NO 消去作用を示さなかった. 以上より, ヤーコン茶葉熱水抽出物にリポキシゲナーゼ阻害や NO 産生抑制が見出されたことから, 同抑制作用を機序とする抗炎症作用に注目した応用利用に期待がもたれる.

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