Determination of integral stereoselectivities of native and recombinant lipases from *Cordyceps militaris* on tricaprylin with kinetic modeling

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ABSTRACT

Lipase (EC. 3.1.1.3), an enzyme which catalyzes the hydrolysis of triacylglycerols, is utilized in various industries due to its regio- and stereoselectivity. The concept of integral stereoselectivity was suggested to exactly describe these selectivities on all acylglycerols during hydrolysis. Recently, three lipase isoforms and recombinant lipase from *Cordyceps militaris* (CML 1–3 for each isoform and rCML for recombinant) were identified, and characteristics of lipase activity and substrate specificity were evaluated. In this study, the integral stereoselectivities of CMLs were investigated to identify the exact stereochemical characteristics. CML 1–3 extracted from lyophilized *C. militaris* and rCML expressed in *Pichia pastoris* were purified. The integral stereoselectivity of each CML was evaluated using tricaprylin, on which all CMLs showed lipase activity. For analysis of the tricaprylin hydrolysis, HPLC-UV/ELSD system equipped with a chiral stationary phase column, CHIRALPAK AY-3, was employed. To quantitatively describe the integral stereoselectivity of CMLs, an interface-based kinetic model considering the Ping-Pong Bi-Bi mechanism, acyl migration, and interfacial characteristics was utilized to fit the experimental results. The estimated kinetic parameters obtained from the kinetic modeling indicated that CML 1, 2, and rCML exhibited *sn*-1,3 selectivity on tricaprylin, while CML 3 displayed *sn*-2 selectivity. These results suggest CMLs as potential lipases for the production of diverse structured lipids with high value in the lipid industry such as structured lipids containing medium-chain fatty acids.

OBJECTIVES

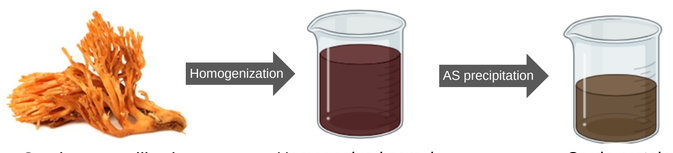
- Production and purification of lipases from *Cordyceps militaris* (CMLs)
- Analysis of tricaprylin hydrolysates by HPLC-UV/ELSD
- Determination of integral stereoselectivities of CMLs on tricaprylin

MATERIALS and METHODS

Production and purification of CMLs

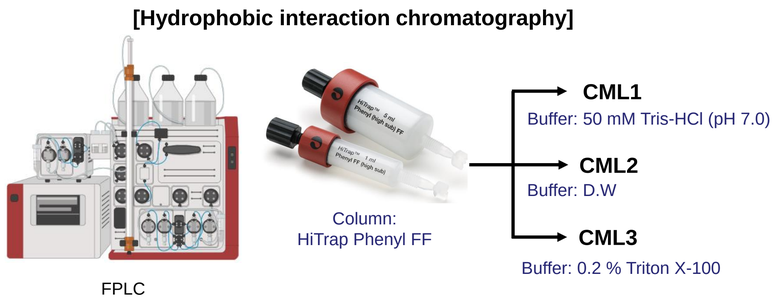
Wild-type CML (CML1, 2, and 3)

- **Protein extraction**



- **Purification**

[Hydrophobic interaction chromatography]

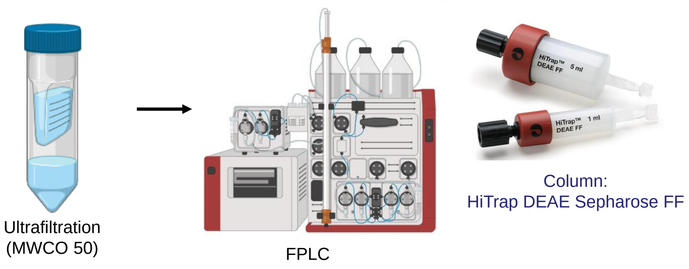


Recombinant CML (rCML)

- **Protein expression**

- **Purification**

[Anion exchange chromatography]



Analysis of tricaprylin hydrolysis

Lipase assay

Table 1. Reaction conditions

Conditions	
System	Emulsion
Surfactant	Gum arabic (5% Conc.)
Temperature	40˚C
pH	7.0
Stirring speed	500 rpm
Total volume	5.2 mL

[Experiment steps]

Starting reaction by adding 0.2 mL enzyme solution

↓

Sampling 200 µL and mixing with 400 µL of *n*-hexane

↓

Voltexing for 1 min and collecting 200 µL of hexane

↓

Injecting of 10 µL to HPLC system

HPLC-UV/ELSD system

CHIRALPAK AY-3 column

Amylose tris (5-chloro-2-methylphenylcarbamate) coated on silica gel

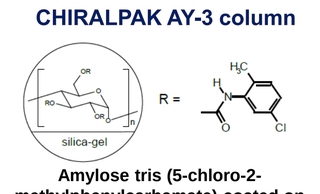


Table 2. HPLC system conditions

Conditions	
Flow rate	0.5 mL/min
Column temperature	30˚C
Mobile phase	*n*-hexane:ethanol: trifluoroacetic acid (90:10:0.1, v/v/v)
Injection volume	10 µL
Wavelength	215 nm
Gas flow rate	1.7 L/min
Nebulizer/drift tube temperature	46˚C
Gain	2

Determination of integral stereoselectivity

- **Integral stereoselectivity**

Tricaprylin —CMLs→ 1,2-*sn*-dicaprylin, 2,3-*sn*-dicaprylin, 1,3-*sn*-dicaprylin

Peak areas of DCs → Concentrations of DCs (Calibration curve)

[Selectivity]

- *sn*-1 selectivity: $\frac{2,3-sn-\mathrm{DC}}{1,2-sn-\mathrm{DC}+2,3-sn-\mathrm{DC}+1,3-sn-\mathrm{DC}}$
- *sn*-2 selectivity: $\frac{1,3-sn-\mathrm{DC}}{1,2-sn-\mathrm{DC}+2,3-sn-\mathrm{DC}+1,3-sn-\mathrm{DC}}$
- *sn*-3 selectivity: $\frac{1,2-sn-\mathrm{DC}}{1,2-sn-\mathrm{DC}+2,3-sn-\mathrm{DC}+1,3-sn-\mathrm{DC}}$

RESULTS

Purification of wild-type CML

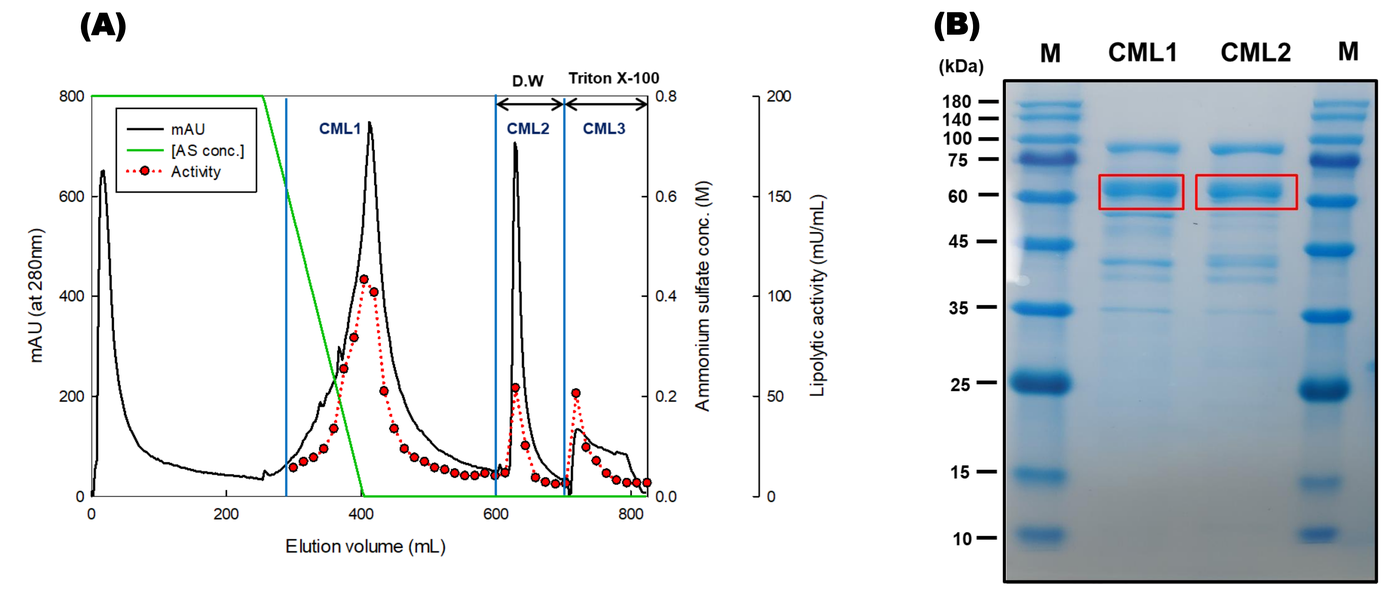


Fig. 1. (A) Elution profile of lipases from *C. militaris* by hydrophobic interaction chromatography. Crude extract from *C. militaris* was resolved with a linear gradient elution of ammonium sulfate (0.8-0 M). Deionized water and 0.2% Triton X-100 were applied to elute hydrophobic proteins interacting with the column. The lipolytic activity of each fraction was evaluated with *p*-NPP. (B) SDS-PAGE analysis of purified CMLs.

Purification of recombinant CML

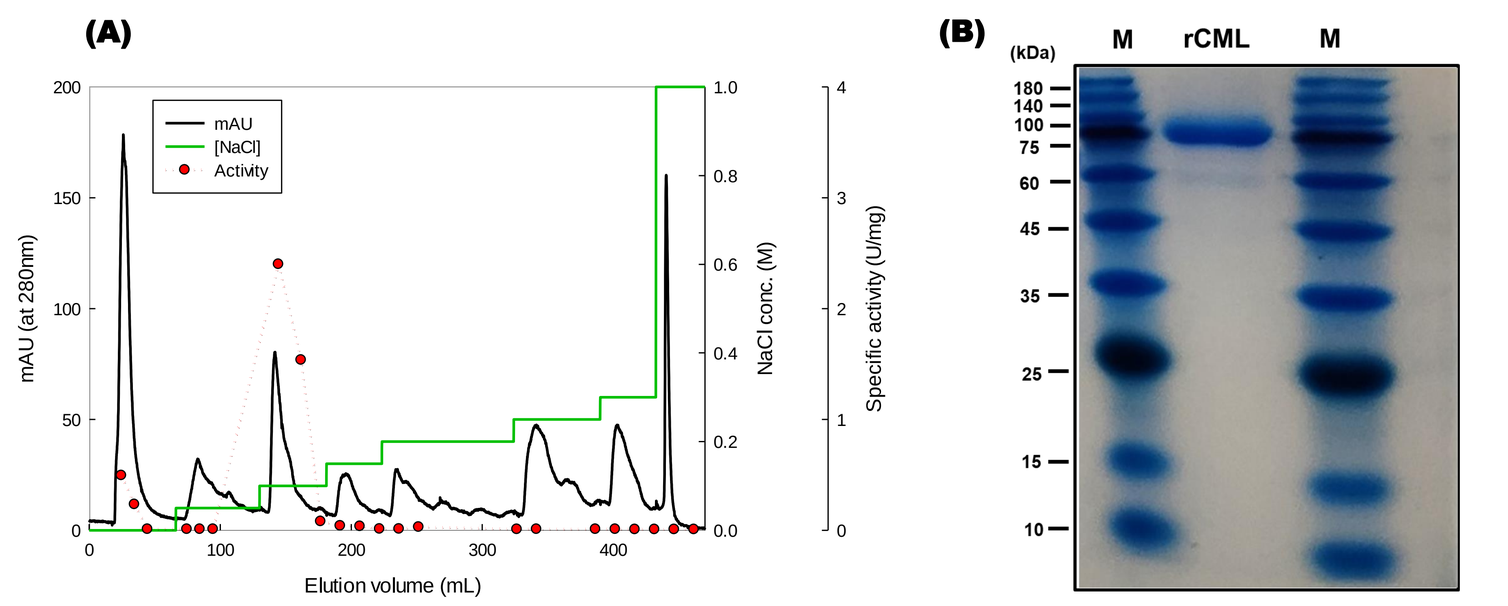


Fig. 2. (A) Purification of recombinant lipase from *C. militaris* (rCML) by anion exchange chromatography. Chromatogram of rCML on DEAE Sepharose FF column using stepwise elution. The lipolytic activity of each fraction was evaluated with *p*-NPP. (B) SDS-PAGE analysis of purified rCML.

Chromatographic separation of tricaprylin hydrolysates

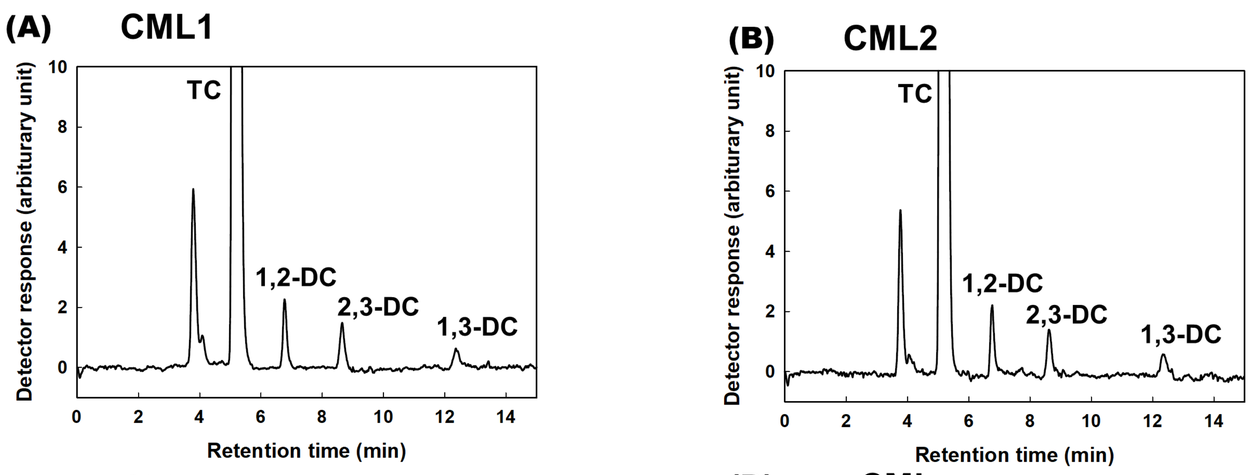


Fig. 3. Chromatograms of reaction mixtures of tricaprylin hydrolysis catalyzed by (A) CML1, (B) CML2, (C) CML3, and (D) rCML at 8 h.

Integral stereoselectivities of CMLs on tricaprylin

Table 3. Integral stereoselectivities of CMLs calculated by ratio of produced dicaprylin isomers at 8 h

Lipase	CML1			CML2			CML3			rCML		
Concentration (µM)	2,3-DC	1,3-DC	1,2-DC	2,3-DC	1,3-DC	1,2-DC	2,3-DC	1,3-DC	1,2-DC	2,3-DC	1,3-DC	1,2-DC
	2.3	0.6	2.4	1.9	0.6	2.4	20.8	2.5	34.4	1.6	0.8	1.15
Selectivity (%)	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3
	42.2	11.2	46.6	39.4	11.6	49.0	36.0	4.3	59.7	22.7	32.2	45.1

CONCLUSION

- ❖ Integral stereoselectivities of native and recombinant lipases from *Cordyceps militaris* (CMLs) on tricaprylin, a representative medium-chain triacylglycerol, were determined by chiral HPLC-UV/ELSD system.
- ❖ Through integral stereoselectivities of CMLs, CMLs can be utilized for production of various medium-chain triacylglycerol-based structured lipids.

ACKNOWLEDGEMENT

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