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Establishing a new quantitative method for *Fusarium* hydrophobins using LC/MS/MS

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【Introduction】

Hydrophobin (HFB), an amphiphilic low-molecular protein produced by filamentous fungi that infect barley and malt, is main cause of beer primary gushing. HFB produced by *Fusarium* fungi are especially known for their tendency to induce gushing. It has been reported that, ~3 μg/L of such HFB in beer can cause gushing[1]. Previously, the ELISA method using polyclonal antibody was reported[1]. In order to evaluate HFB content more accurately, highly specific quantification of HFB is required. Using UPLC-triple quadrupole tandem mass spectrometry (LC/MS/MS), we established a highly specific and sensitive analysis that quantified the target HFB produced by *Fusarium graminearum.*

【Method】

< HFB analysis using LC/MS/MS >

[The feature]

Since quantification is based on the mass of the peptide fragments specific to the target protein, highly sensitive and specific quantification is possible.

1. Selection of target fragments

Target protein — We chose HFB produced by *Fusarium graminearum.*

<Because>
A typical species that infects barley has strong ability to induce gushing.

Based on the amino acid sequence of the target protein, the peptide fragments with a specific and strong signal were selected.

[Peptide Selection Criteria[2]]

- Length between 6 and 16 amino acids
- No posttranslational modifications
- No single nucleotide polymorphism
- Containing one of leucine, isoleucine, valine, alanine or proline residue. etc.

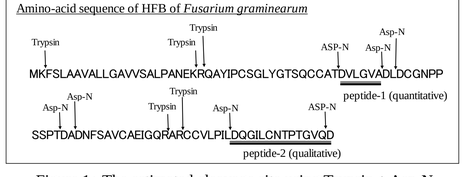

Figure 1: The estimated cleavage site using Trypsin + Asp-N

2. LC/MS/MS analysis

① Sample preparation

[Beer]
Sample 20 μL
↓ ← Add I.S. (Stable isotope labeled-peptide)
← Dithiothreitol
30 min (56ºC)
↓ ← Iodoacetamide
30 min
↓ ← Trypsin + Asp-N
24 h (37ºC)
↓
Clean up (SPE C18)
↓
LC/MS/MS analysis

[Malt]
5 g of the fine flour
↓ ← 60% Ethanol, 50 mL
Extraction (30ºC, 200 rpm, over night)
↓
Centrifugation
↓ (Supernatant fraction)
The same method of beer

② LC/MS/MS conditions

[UPLC analytical conditions]
LC : SHIMADZU Nexera X2
Column : L-column2 ODS (2.1 mm I.D. x 50 mm, 2 μm, CERI)
Mobile phase : A : 0.1% Formic acid B : Acetonitrile
Flow rate : 0.3 mL/min
Gradient min (A/B) : 0 (95/5), 30 (50/50), 32 (5/95), 35 (95/5)
injection : 20 μL

[MS analytical conditions]
MS : Sciex API 4000
Ionization : ESI Positive

Table 1: MS analytical conditions

	Precursor ion (m/z)	Product ion (m/z)	DP (V)*	CE (V)*	CXP (V)*
Peptide-1 (Quantitative)	573.4	484.5	81	23	20
		385.2		29	14
Peptide-2 (Qualitative)	759.8	243.9	96	47	6
		301.2		47	10

*) DP (Declustering potential), CE (Collision energy), CXP (Collision cell exit potential)

< Gushing Test >

We prepared a microbrew beer based on the method proposed by Vaag *et al.*[3] and conducted a gushing test according to method-A[4] proposed by Amaha *et al.*

【Result】

1. Validation of the method for quantitative determination of peptide-1

Table 2: Validation of beer analysis

Linearity	Detection limit* (μg/L)	Quantitation limit* (μg/L)	Repeatability (%)	Interday variation (%)
0.9998	0.8	2.0	4.8	5.1

Table 3: Validation of malt analysis

Linearity	Detection limit* (μg/g malt)	Quantitation limit* (μg/g malt)	Repeatability (%)	Interday variation (%)
0.9990	0.05	0.12	8.0	11.5

*) Converted to HFB concentration < Repeatability : n=6 Interday variation : n=6, 3days >

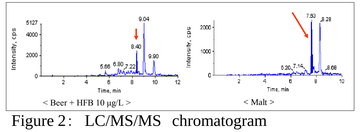

Figure 2: LC/MS/MS chromatogram

- The analytical sensitivity and precision of peptide-1 were satisfactory for both beer and malt.

※ The HFB-producing species detected by this method

Table 4: The HFB-producing species estimated by the combination of peptide-1 and peptide-2

	The HFB-producing species
Detection of only peptide-1	*Trichoderma reesei* , *Trichoderma virens* , *Neurospora crassa* , *Claviceps fusiformis* , *Cryphonectria parasitica* etc.
Detection of only peptide-2	*F. poae* , *F. oxysporum* *
Detection of both peptide-1 and peptide-2	*F. graminearum* , *F. culmorum* , *F. verticillioides* , *F. fujikuroi* , *F. pseudograminearum* , *F. oxysporum* **

* HFB Name : FOQG_13743
** HFB Name : FOVG_15013, FOXB_00066, FOWG_12166, Trihydrophobin

Table 5: Typical species that causes beer gushing not detected by this method

The HFB-producing species
F. avenaceum , *Nigrospora* sp., *Penicillium* sp., *Aspergillus* sp. etc.

BLAST search results for the sequences of species registered in the NCBI database.
Parts in the red : Producing species that strongly induces gushing in beer [1)5)].

- Detection of peptide-1 indicates the possibility of molds such as *Fusarium* and *Trichoderma*.
- Detection of both peptide-1 and peptide-2 allows for the estimation of six species belonging to the genus *Fusarium*.
- Detection of only peptide-2 allows for the estimation of two species belonging to the genus *Fusarium*.

2. The results of HFB analysis of malt sample

Table 6: The results of HFB analysis of malt sample

Sample No.	Area	Gushing Test (mL/200 mL)	Peptide-1 HFB (μg/g malt)	Peptide-2 Detection
Sample-1	Europe	101	0.57	○
Sample-2		45	trace	○
Sample-3		40	0.97	○
Sample-4		20	0.43	○
Sample-5		11	0.20	
Sample-6		7	N.D.	
Sample-7		7	0.20	
Sample-8		3	0.39	○
Sample-9		1	0.52	○
Sample-10		0	0.14	
Sample-11		0	N.D.	
Sample-12		0	N.D.	
Sample-13		0	trace	
Sample-14	North America	96	N.D.	
Sample-15		71	0.24	
Sample-16		19	N.D.	
Sample-17		0	N.D.	

N.D. : Lower than the detection limit (0.05 μg/g malt)
trace : Lower than the quantitation limit (0.12 μg/g malt)
○ : Detection of peptide-2

- Presence of target HFB was confirmed through detection of peptide-1 and peptide-2 in the malt samples.
- European malts with peptide-1 tended to exhibit gushing. Some types of North American malts exhibited gushing in the absence of target HFB.
- By addition of other target HFBs, this method will be able to detect a larger variety of HFBs produced by fungi that induce gushing.

【Conclusion】

1. Using LC/MS/MS, we established a highly specific and sensitive analysis that quantified the target HFB produced by *Fusarium graminearum.*
2. Measurement of peptide-1 and peptide-2 enabled the detection of HFB-producing species.
3. European malts with peptide-1 tended to exhibit gushing.
4. This method allowed for the quantitative analysis of HFB in beer and malt, and the analytical results aided in the estimation of the HFB-producing species. This method can be used to improve the quality of beer and malt associated with primary gushing.

【References】
(1)Sarlin, T., *et al.*; *J. Inst. Brew.*, 111(2), 105–111, 2005
(2)Kamiie, I., *et al.*; *Pharm. Res.*, 25, 1469-1483, 2008
(3)Vaag, P., *et al.*; EBC Congress, 155-162, 1993
(4)Amaha, M., *et al.*; *Tech. Quart. MBAA*, 15, 15-22, 1978
(5)Sarlin, T.; VTT Science, 13, 2012