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Applications of a novel hydrolysis system for deconvolution of cyclotides on a bead and super rapid amino acid analyses

MP577

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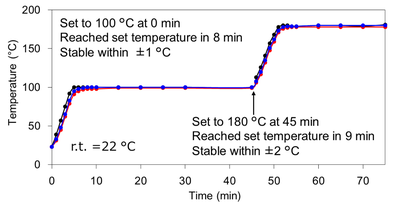
Summary

Recently demands on discovery and QC in drug development are increasing, hence facile and rapid hydrolyses of peptides and proteins are required. We have constructed a novel hydrolysis system which adapted to wide range of temperature without leakage of HCl fume. Two applications of AHST-16 are shown.

(1) A library consisting of cyclic octa-peptides as capturing molecules consisting of 24 natural and non-proteinogenic amino acids on the manner of "one peptide on one bead (OPOB)" focusing on drug discovery. The system are applied for rapid deconvolution of OPOB using MS/MS, of which methods has been improved based on the previously reported. (2) As an another application, super rapid amino acid analyses was performed, while 16 samples could be analyzed with rapid hydrolyses followed by the rapid determination by LC/MS using unique separation columns in a single day. Thus AHST-16 is useful for deconvolution immobilized peptides and for high throughput amino acid analyses in QC of peptides and proteins.

AHST-16

Hydrolysis system, reactions under sealing, higher efficiency & labor saving in acid hydrolysis:



Improved sensing mechanism allows rapidly reaching to the setting temperature of each position

Advantages

(1) Easy vial sealing **no-use by "open flame"** *ie.* without burners; (2) For various chemical processes under sealed conditions. ; (3) Easy operation without skills; (4) Contamination free, semi-microscale, one touch sealing (just ca 1 min) after reaction excess reagents can be rapidly removed by nitrogen stream for ca 30 sec using the same apparatus.

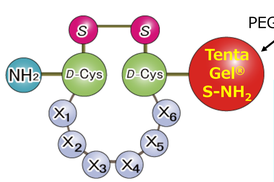
Part 1

Improved deconvolution method for cyclic peptides immobilized on Gel-type beads constructed for drug discovery tool

OPOB* has been prepared for drug discovery [*one single peptide immobilized on one bead, **LIBRARY of cyclic octa-peptides** (involving two ***D***-Cys) immobilized on a gel-type resin (TentaGel®) which allows bioassays in an aqueous media

High quality cyclic peptide-beads as capturing molecules constructed with 24 amino acids and cyclized with two ***D***-Cys = **Diversity 24^6 = ca. 200 millions;** No inter-molecular disulfides; ca 100 μm particle; Peptide loading *ca* 80 pmole/bead; 2.3 million beads/g Selected single bead are deconvoluted by partial hydrolysis with MS/MS

The present report is the improved method from previously reported: Nokihara et. al., *Amino Acids*, 48, 2492-2499, 2016. DOI 10.1007/s00726-016-2269-1



PEG-grafted polystyrene Beads: 90 μm, 0.27 mmol/g

24^6 = *ca* 200 million Xn = 19 natural and 5 **non-proteinogenic amino acids (Cha, Hyp, Nle, Nva, Phg)**

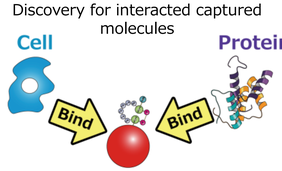
Construction of OPOB

Split & combine method, Deprotection and coupling are carried out using PetiSyzer® with LibraTubes®; On-resin cyclization in 10% DMSO (pH6.0) at room temperature for 4 days.
Detail: Nokihara et. al., *Amino Acids*, 48, 2492, 2016

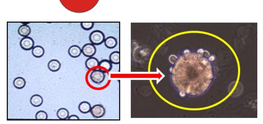
OPOB: available from HiPep Labs (http://hipep.jp/eng/?p=751) as research products and/or Nakarai Tesque US.

OPOB is a useful tool for drug discovery

Discovery for interacted captured molecules



Beads recognized cell surface proteins

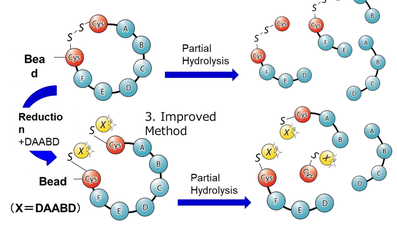


Screening ➡ Deconvolution

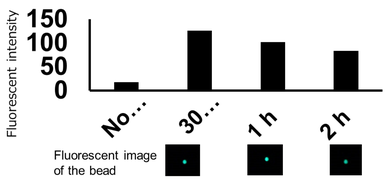
Partial hydrolysis with 5.7N HCl at 60 °C for 3 h by the use of the AHST-16 ➡ LCMS／MALDI-TOF-MS/MS

Problems in deconvolution

1. LC-MS of partial hydrolysate of cyclotides on beads gave difficulties to identify the corresponding peaks
2. Termini were unclear since target was cyclic



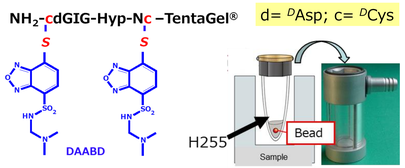
Fluorescent intensity changes after hydrolysis of the labeled bead during hydrolysis (UV 375 nm)



Fluorescent intensity (UV375 nm) of the labeled bead was gradually decreasing depends on duration of hydrolysis = the immobilized peptide was gradually liberated (detached from a bead).

Hydrolysate: Analyzed by LC-MS [LC: Agilent 1100+ MS: HCTultra (IT-MS, Bruker)]

Cysteine residues at both termini of cyclotide immobilized on bead were derivatized with DAABD*-Cl (fluorescent labelling agent) by the one-pot reaction (10 min)

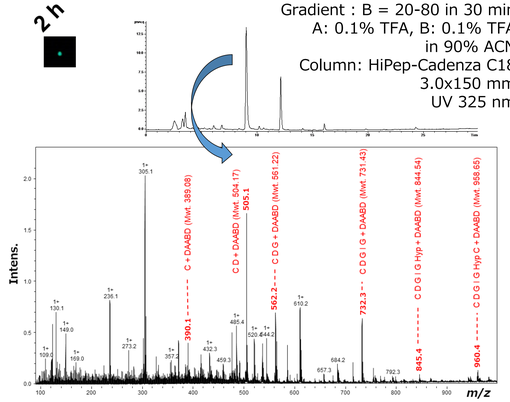


4-[2-(Dimethylamino)ethylamino-sulfonyl]-7-chloro-2,1,3-benzoxadiazole

Methods

1. A bead was reduced and labeled in 0.83 mM tris(2-carboxyethyl) phosphine), 0.14 M DAABD-Cl
2. Washed with MeOH, H_2O
3. Resulted bead was partially hydrolyzed in a vial H255 with 5.7 N HCl (20 μL) under sealing at 60 ℃ for 30 min, 1 h, 2 h using AHST-16

Flow rate : 0.3 mL/min
Gradient : B = 20-80 in 30 min
A: 0.1% TFA, B: 0.1% TFA in 90% ACN
Column: HiPep-Cadenza C18 3.0x150 mm
UV 325 nm



LC-MS of hydrolysate of the above DAABD-peptide: was labeled with DAABD (5.7 N HCl for 2 hrs)

Conclusion of Part 1

By reducing and derivatizing for Cys-cyclotides with DAABD, the sensitivity on LC-MS was enhanced. Fluorescent labeling facilitated identification of the fragments generated by the partial hydrolysis, and sequence analyses were efficiently improved.

① Cyclic peptide beads were reductively derivatized with DAABD-Cl. The progress of the hydrolysis has been shown as fluorescence intensities of the bead was decreased. Thus the peptide on a bead was fragmented and released by the hydrolysis.

② The peptide has been partially decomposed by hydrolysis at lower temperature for shorter time.

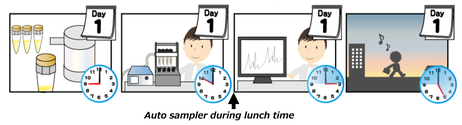
③ In the case of a peptide consisting of Cys at both termini, fluorescent labeling of Cys residues resulted higher efficient deconvolution.

④ Hydrolysate of the OPOB-bead for 2hours several fragment peaks were observed at LC-MS analysis. The mass indicated in the spectrum gave agreement with the theoretical value of the corresponding fragment generated by hydrolysis.

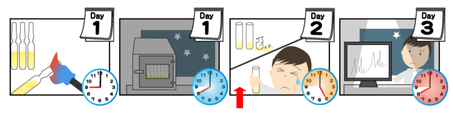
Part 2

High throughput Amino Acid Analysis by the Novel System without any derivatization ➡ up to 16 Samples in a single day

Smart analyses Labor saving



Conventional and previous manner



Vial broken, HCl fume destroys oven…Not completely hydrolyzed……

Hydrolysis with HCl (5.7 N) & TFA (2:1 v/v) at 165 ℃ for 25 min [1], AA-composition can be analyzed by Mass Chromatograms ➡ Racemization caused [2]

The present column can**not discriminate *D* & *L*-isomers**.

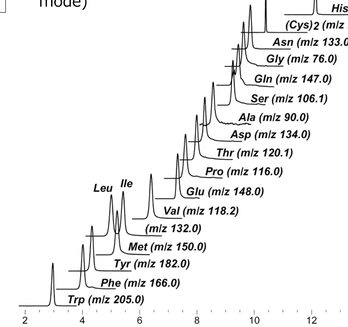
Throughput depends on the column length	
50 mm	ca 10 min
100 mm	ca 15 min
150 mm	ca 20 min

Detection by a Mass Spectrometer, *Not UV*
Lower cost MS, such as single Q-pole MS is enough

A typical separation profiles of standard amino acids without derivatization:

Column: Intrada Amino Acid 3 id x 100 mm

Flow rate 0.6 mL/min, 35 ℃; detection by LCMS (ESI positive mode)

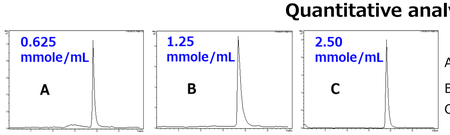


Eluent:
A = ACN, THF, 25 mM NH_4COOH, HCOOH (9:75:16:0.3 v/v);
B = ACN, 100 mM NH_4COOH (20:80 v/v);
Gradient: B% = 0 (0-3 min), 0-17 (3-9 min), 100 (9-16 min)

Quantitative analyses by a LC-MS

AA	regression equation	R^2
F	y = 1E+09x+7E+08	0.9613
I	y= 9E+07x+2E+08	0.7582
L	y= 1E+08x+1E+08	0.9988
V	y= 1E+08x+7E+07	0.9554
E	y= 1E+08x+4E+07	0.9833
D	y= 5E+07x+1E+07	0.9770
N	y= 6E+07x+3E+07	0.9487
H	y= 2E+08x+5E+07	0.9979
T	y= 6E+07x-3E+07	0.9746
G	y= 1E+07x+3E+06	0.9817
Q	y= 2E+08x-2E+08	0.9509
C	y= 7E+07x-4E+07	0.9997
R	y= 4E+08x-3E+08	0.9097
S	y= 5E+07x-3E+07	1
P	y= 4E+08x-3E+06-8	0.9445
K	y= 2E+08x-1E+08	0.9411
M	y= 4E+08x-4E+08	0.9257

Quantitative analyses by a LC-MS



	Area
A	2721573
B	5004978
C	697261246

Conditions
Column: Intrada AminoAcid (150 x 3 id mm)
Flow Rate: 0.6 mL/min
Gradient :B%=20 (0-5 min), 35 (5-11 min), 100 (11-20 min)
A: 0.3% HCOOH in ACN, B: 100 mM Ammonium formate/ACN = 80/20, at 40℃; HCTplus, Bruker

Conclusion of Part 2

Current difficulties in High Throughput analyses by MS ➡ Determination: Gln *vs* Lys; Leu *vs* Ile; N- & C-termini : free or modified?; Gln & Lys give similar m/z, Leu Ile give the same *m/z;* High resolution MS (= costly instrument) + contaminants causes noise & disturbing interpretation

① High throughput amino acid analysis was performed by **AHST-16**, with the column **Intrada Amino Acid**, and **LC-MS**.

② Acid hydrolysis: **HCl:TFA = 2:1 v/v (166℃ for 25 min) ➡** Throughput at least **16 samples in a day**

③ The present AA-analysis gives useful information without costly instruments, such **as Amino acid analyzers**.

The KEYs for MS-*de novo* sequencing
①N-terminus: acetylation gave peak shift +42 Da
②C-terminus: methyl esterification gave peak shift +42 Da