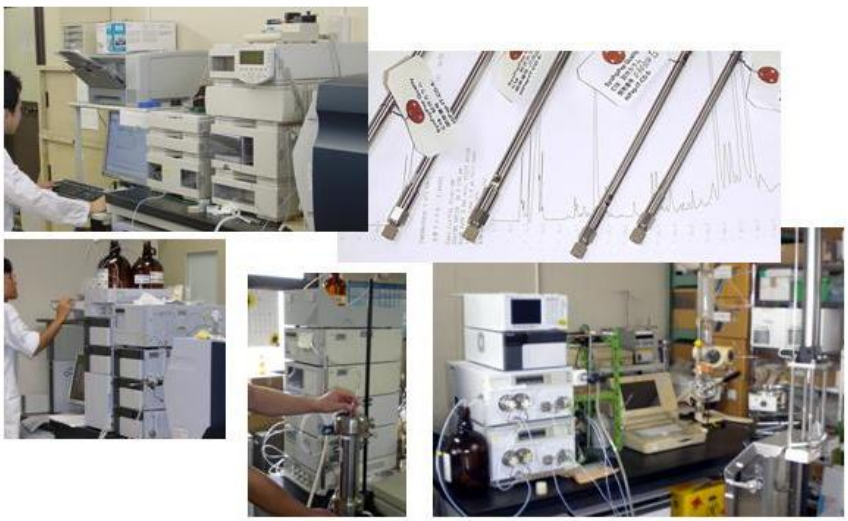
Handbook of HiPep-Columns for Proteins & Peptides

Catalog and Technical Notes



HiPep Laboratories URL : www.hipep.jp info@hipep.jp
Tel : 075-813-2101 Fax : 075-801-0280
from abroad voice +81-75-813-2101, FAX +81-75-801-0280

HiPep Columns: Peptide focused high resolution Separation
HiPep-Cadenzaz, a C18 reverse phase packing

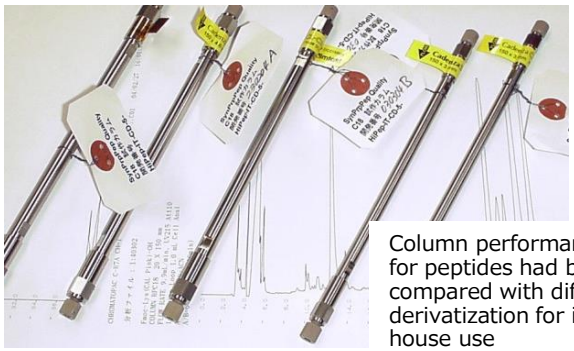
BACKGROUND The founder of HiPep Laboratories, Dr. Nokihaara has continued to research peptides and their derivatives since 1973. In the meantime, he anticipated HPLC which was introduced in bio-research field, and invested a lot of efforts to purification and analysis. HiPep mainly used a wide variety of C18 reversed-phase silica gel columns for synthetic peptides generated by the SPPS which have different molecular-weights up to 15KDa, also natural peptides isolated from tissue (organs). After foundation of HiPep Laboratories in 2002 the best packing for peptide analysis, separation, and preparative purification had been found and commercialized listed below.

Purification tests had been carried out with varieties of packing materials with different derivatization and optimized by using specially designed synthetic peptides.

A column sized 20 id X 150 mm can separate around 10 mg of crude peptides at one run. Purification recovery depends on crude materials and generally 30 to 50%.

Important performances for HPLC columns:

- **High resolution, reproducibility, endurance**
- **Easy migration to semi-preparative purification**
- **Higher recovery in preparative purification**



Column performances for peptides had been compared with different derivatization for in-house use

P/N	Purpose	Size (mm)	Flow rate
HC18-375	High-throughput analysis	3.0 id X 75	0.3 mL/min
HC18-4675	High-speed analysis	4.6 id X 75	0.5 mL/min
HC18-2150	LC-MS for peptide-mapping	2.0 id X 150	0.15～0.2 mL/min
HC18-3150	Solvent-saving analyses	3.0 id X 150	0.5 mL/min
HC18-46150	Regular analysis	4.6 id X 150	1.0 mL/min
HC18-20150	Semi-preparative purification	20 id X 150	10～20 mL/min
HC18-10250	Tissue extraction, prep.purification	10 id X 250	2～3 mL/min
HC18-20250	Prep. purification	20 id X 250	10～20 mL/min
HC18-28250	Prep. purification	28 id X 250	20～40 mL/min

All columns above have greater performances than the others in theoretical plate number, resolution, residual silanol, acid resistance and lower nonspecific adsorption. For designing, we gave priority to highly-efficient separation for peptide and related materials. All analytical columns are LC-MS-suitable, while HiPep Labs routinely use those columns for own R and D as well as contract research.

Precautions for column handling

Please avoid excessive mechanical shock.

When you connect columns: There is a mark of **FLOW** on the column's label. Please connect the column in the direction of eluent flow. When you use a wrench to set tube-in-tube, please put the wrench at the hexagonal part of its end-fitting top. Do not put the wrench at a slit on column's pipe.

Before use insoluble material must be removed from sample solution by centrifugation in advance, and have to be filtered with membrane-filters of 0.45 μm or 0.2 μm. Floating materials or precipitates may cause pressure increase trouble by clogged columns.

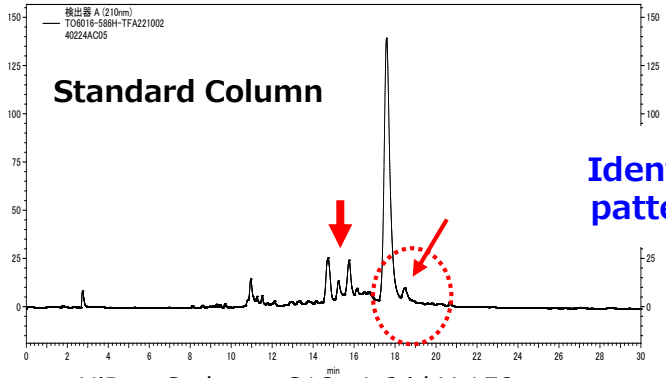
Normal operation pressure: approximately **20 MPa (maximum)**. In consideration of solvent's viscosity, please set flow volume not to exceed maximum pressure.

Appropriate range of column temperature is **15 - 65 ℃.** Please set columns temperature, in consideration of peak shape, retention capacity and separation characteristic.

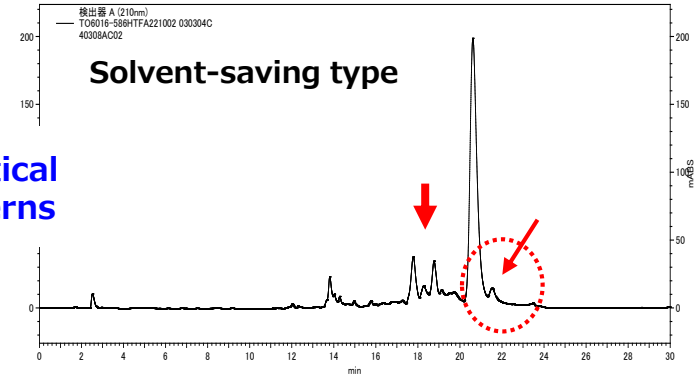
Generally silica-based columns should be used between **pH 2 - 7.** Please set pH according to the purpose of your analysis. When use in alkaline-condition in the long term, column life time may shorten. Any case column life can be changed depending on the analyses conditions such as organic solvent concentration, pH adjuster composition, temperature and structure of analytes. In case of long-term storage of columns after use, please make sure to displace them in column storage solvent. Long-term storage in alkaline or acidic conditions must be avoided.

Column-wash: please remove analytes completely by higher concentration of the organic solvent. After analyses, columns have to be stored by displacing in approximately 60% aqueous acetonitrile or approximately 70% aqueous methanol solution, tightly closed and store in room temperature.

Next page show the resolution comparison. The key issue in discovery from natural resources: resolution of peaks patteren

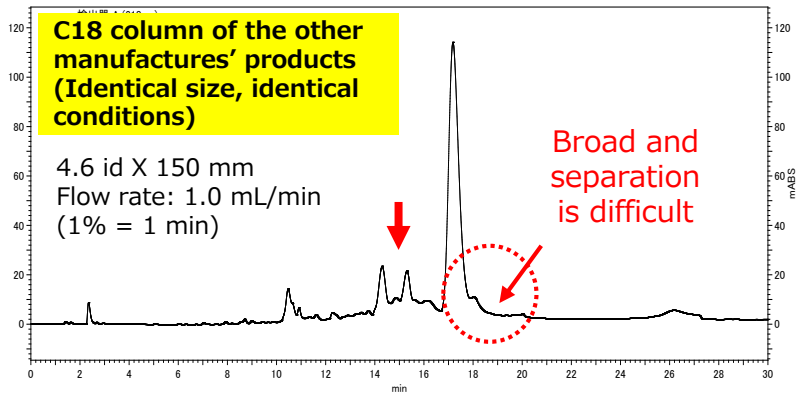


Identical patterns



HiPep-Cadenza-C18, 4.6 id X 150 mm
Flow rate: 1.0 mL/min (1% = 1 min)

HiPep-Cadenza-C18, 3.0 id X 150 mm
Flow rate: 0.5 mL/min (1% = 1 min)



Comparison of Efficiency in Separation/ Resolution: Analyte as an example: Solid-phase syntheses peptides before purification mol. weight: approximately 3200 (Linear Peptide with Arg x 4, Lys x 9)
Column Length 150 mm,UV 210 nm, Eluent: A=0.01N HCl, B=0.01 N 90% Acetonitrile, A/B=95/15-65/35 in 30min.

Efficient separation and purification for structured peptides with difficult sequences using HiPep-Intrada

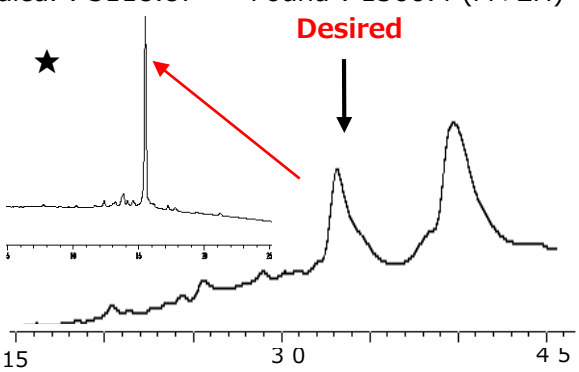
HiPep-Intrada: Wide-pore Reverse-phase Packing Materials

Preparative purification and analysis with HiPep-Intrada

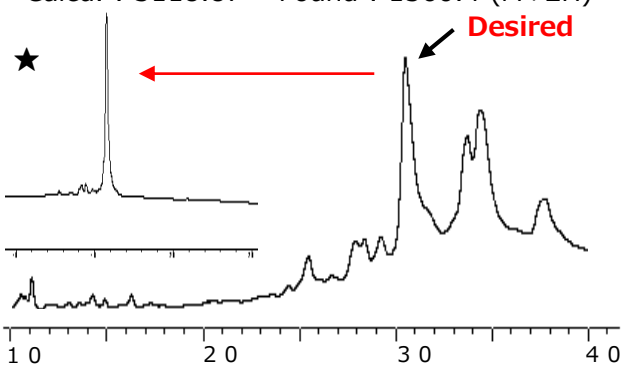
Preparative purification with C18 column (20 id x 150 mm)

Preparative separation by HiPep-Intrada 20 id x 250 mm (60℃): sharper peak

Calcd. : 3118.87 Found : 1560.4 (M+2H)



Calcd. : 3118.87 Found : 1560.4 (M+2H)



★ Analytical conditions of purification samples: HiPep-Intrada 3.0 id×150 mm, Flow Rate: 0.3 mL/min
A= 0.1% TFA. B= 0.1% TFA in 90%ACN Gradient : B= 45-75% in 30 min **60 ℃**

This packing material for HiPep-Intrada has been designed that the resolution in separation is the first priority, thus the column-life is relatively shorter than that of conventional reverse-phase columns. This column is very sensitive against strong acid or alkaline treatment. Following points should be considered in operation to keep longer life.

1.Do not start gradient elution from 100% water.
2.Do not use 100% organic solvents at the end of elution and never washed with 100 % organic solvents.
3.Column Storage: in 30% aqueous methanol solution containing 0.1% acetic acid. Avoid to use 100% water or 70% aqueous methanol solution for storage.
4.HiPep-Intrada often discriminates conformational difference of peptides even the same primary structures (sequence).
In fact, sensitivity in separation of conformational differences is often influenced by isomers or other components existing in analytes. Selection of solvent mixture and their ratio for injection for sample are important.
Reproducibility of peak profiles is also dependent on the dead space between injector and column-inlet. These phenomena are often observed when analyte is structured peptide such as amyloid, Ab-peptides. Ab-peptides were dissolved in DMSO (100%) and injected to sample loop, the analyte was mixed with the initial eluent in the sample loop, many peaks could be observed, of which mass unit were the same. Those peak patterns obtained were split or broadening.

HiPep-Intrada is filled with extremely sensitive derivatized silica.

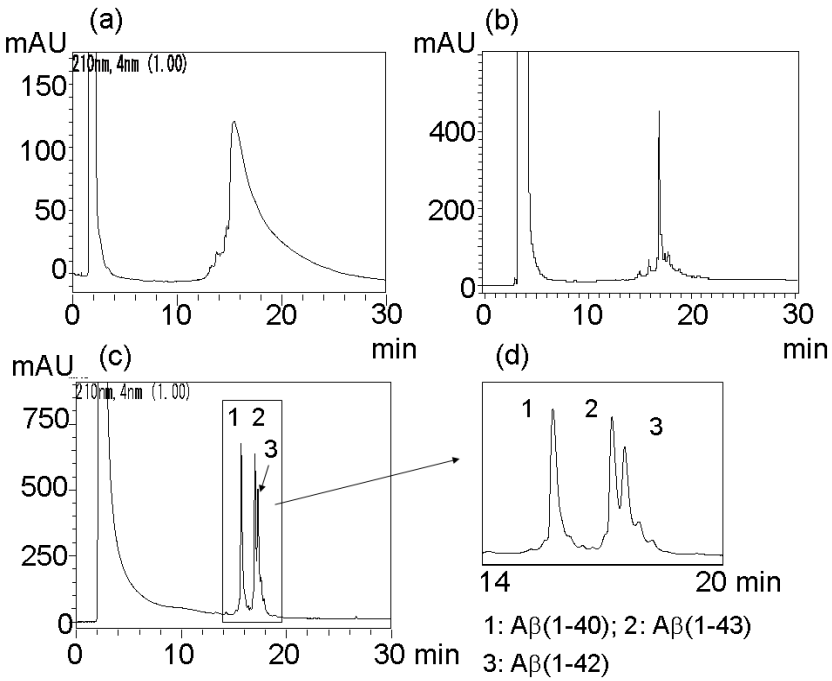
P/N	Purpose	Size (mm)	Flow rate
HiWR-2150	LC-MS for peptide-mapping	2.0 id X 150	0.15～0.2 mL/min
HiWR-3150	Solvent-saving	3.0 id X 150	0.5～0.75 mL/min
HiWR-46150	Regular analysis	4.6 id X 150	0.75～1.25 mL/min
HiWR-10150	Semi-preparative purification	10 id X 150	2～3 mL/min
HiWR-10250	Tissue extraction, prep.purification	10 id X 250	2～3 mL/min
HiWR-20250	Prep. purification	20 id X 250	10～20 mL/min
HiWR-28250	Prep. purification	28 id X 250	20～40 mL/min

Analyses and purificaqtionn of βAmyloid and Prion related peptides by HiPep-Intrada

In recent years, some diseases caused by peptides conformational changes are highlighted as intractable disease. In the research of peptides which have specific secondary/tertiary structures in solutions, single substance and their mixture may have different HPLC profiles due to the some interactions. These phenomena seems to be caused by sample preparation procedures not in the columns. In the case that multiple peptides which have specific structures are combined, interactions among peptides may caused, thus same peptides are eluted at different retention times due to their structural difference. Below indicated analyses of mixtures of Aβ(1-40), Aβ(1-42), Aβ(1-43), hence sample preparations is important.

Hirata, A., Miyajima, M., and Nokihara, K. Separation of peptides having structures and derivatives of mimosine, a non-proteinogenic amino acid, by a novel reverse-phase HPLC column packed with wide-pore silica, J. Liquid Chromatography & Related Technologies, 36, 2960-2967, 2013.

HiPep-Cadenza C18 vs HiPep-Intrada for Aβ peptides



Structured peptides by the conventional C18 colum gives broad peak and difficult to separate, although HiPep-Intrada Give a sharp peak and easyer to characterize

(a) HiPep-Cadenza C18
(b) HiPep-Intrada
(c) HiPep-Intrada for mixture of Aβ
(d) Enlarged (c) at 60℃

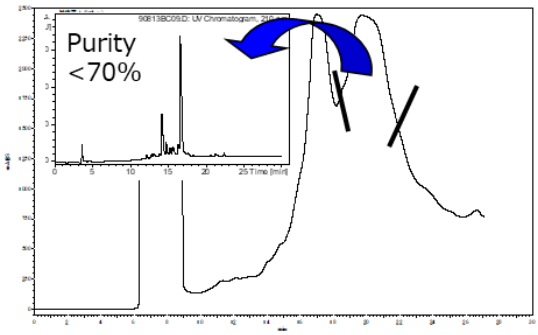
Sample Preparation in Purification for Poorly-soluble Peptides

Peptides which have structures, cause aggregation are/or poor solubility show often broad peak on HPLC elution profiles. In these cases purification is difficult and after purification not only purity but also recovery generally low due to broad peaks. To improve these purification, sample preparation methods have been studied.

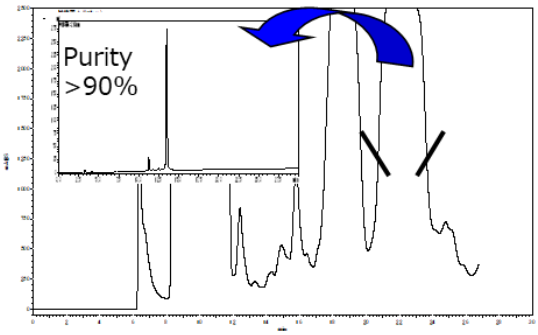
Column: HiPep-Intrada (20 x150 mm), A:0.1% TFA in H_2O; B:0.1% TFA in 90% Acetonitrile, flow rate: 9.9 mL/min, detector: UV at 210 nm, **60 °C**)

Example 1. Peptide A

☆ DMSO preparation

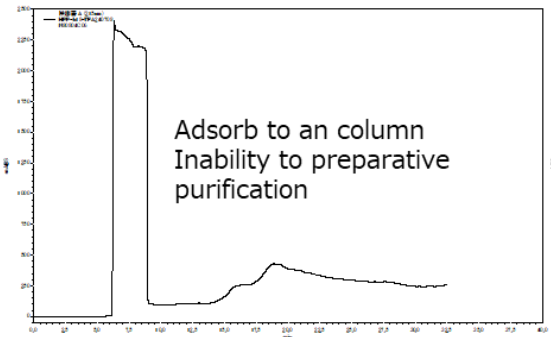


☆Nonafluoropentanoic Acid preparation

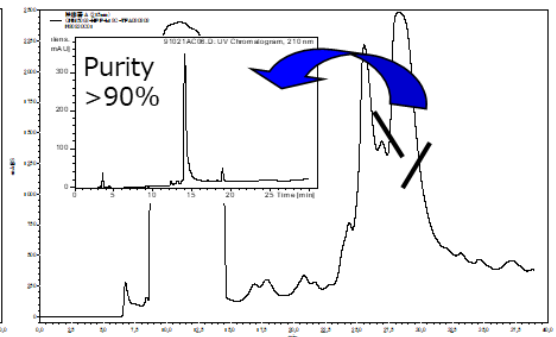


Example 2. Peptide B

☆ DMSO preparation

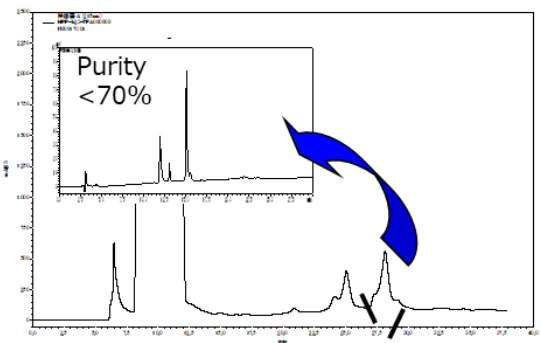


☆Nonafluoropentanoic Acid preparation

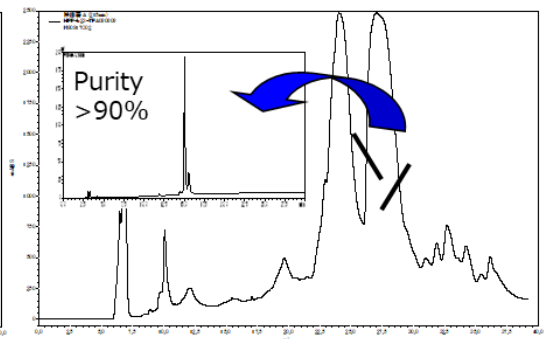


Example 3. Peptide C (peak was not detected in DMSO preparation)

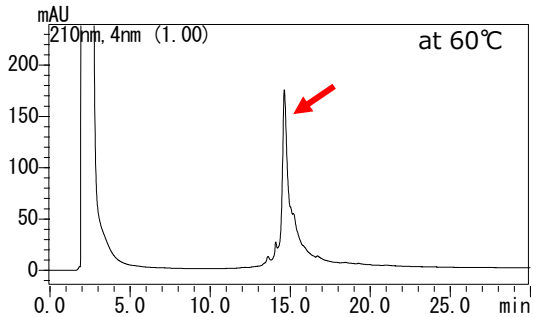
☆Nonafluoropentanoic Acid preparation

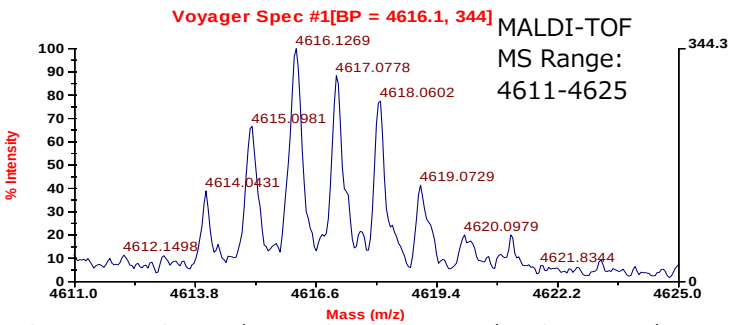


☆HFIP preparation



Analysis of Aβ(1-43) by HiPep-Intrada 3.0 φ x 150 mm





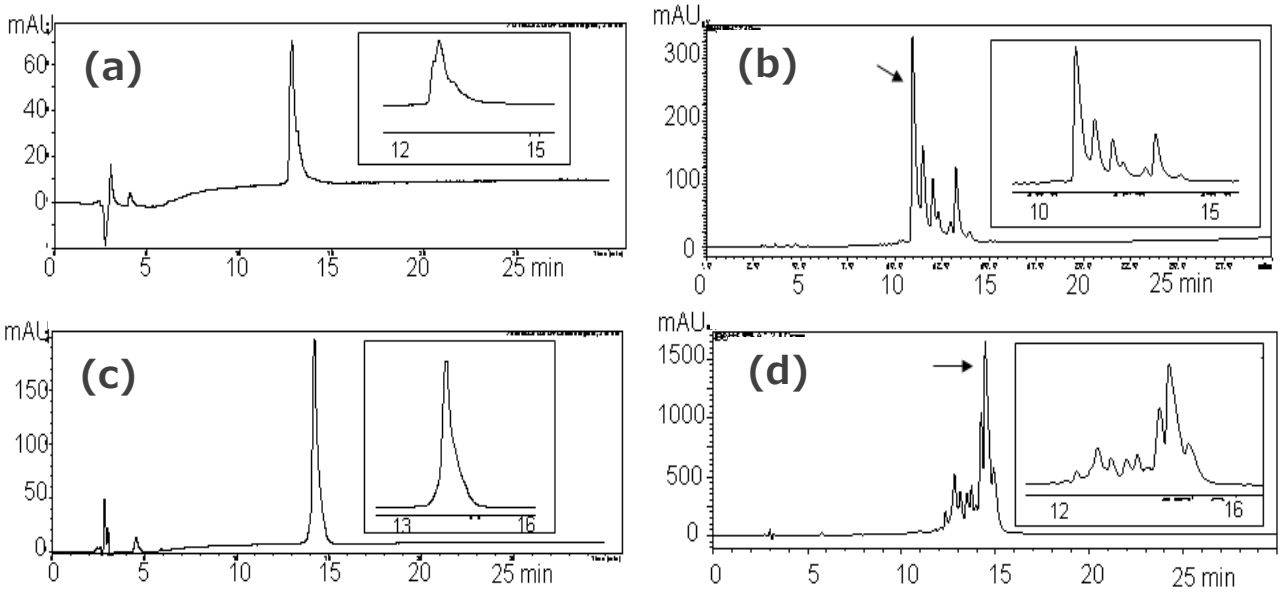
Efficient separation for structured peptides, peptides having aggregation tendency using HiPep-Intrada. HiPep-Intrada discriminate structure of peptides and proteins.

Reference: Peptide Science 2007: S. Aimoto and S. Ono (Eds) The Japanese Peptide Society (2008) page 143-146.

Prion protein related fragment peptides for research

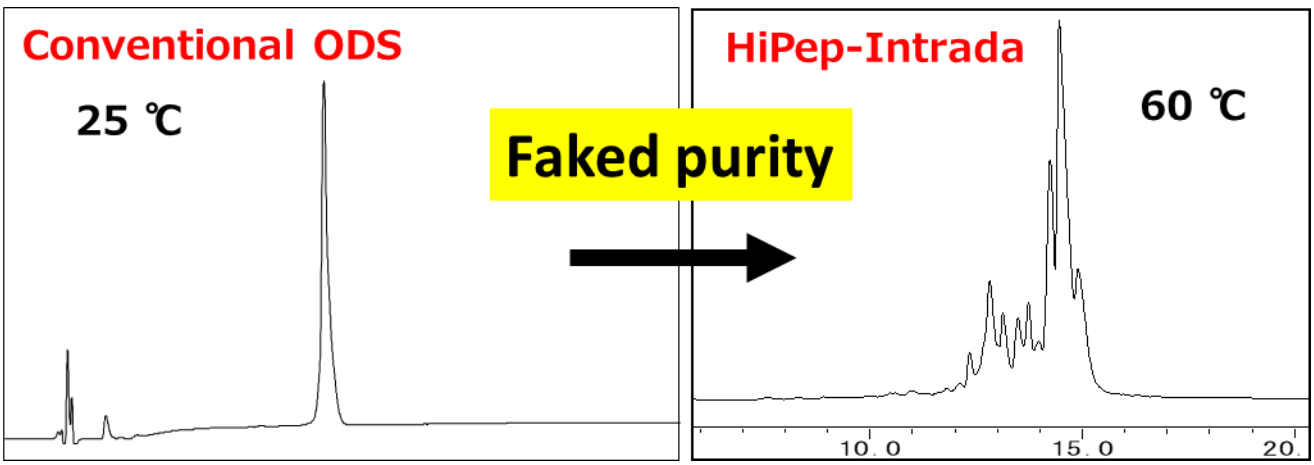
(a), (b); PrP(106-126), (c), (d); PrP[(169-176)+180-195)]

Columns (a), (c); HiPep-Cadenza C18（RT）; (b), (d); HiPep-Intrada （60℃）



HiPep-Cadenza C18 gave single component, although HiPep-Intrada gave multiple components, which was been identifile by LC-MS as multiple MS-signals. Therefore HiPep-Intrada allows components which can not be separated by HiPep-Cadenza C18.

Separation example of HiPep-Intrada *Single peak doesn't mean a single component*



The series of this packing materials has been developed for **difficult and /or aggregated structured peptides** such as βAmyloid Peptides or Prion related peptides

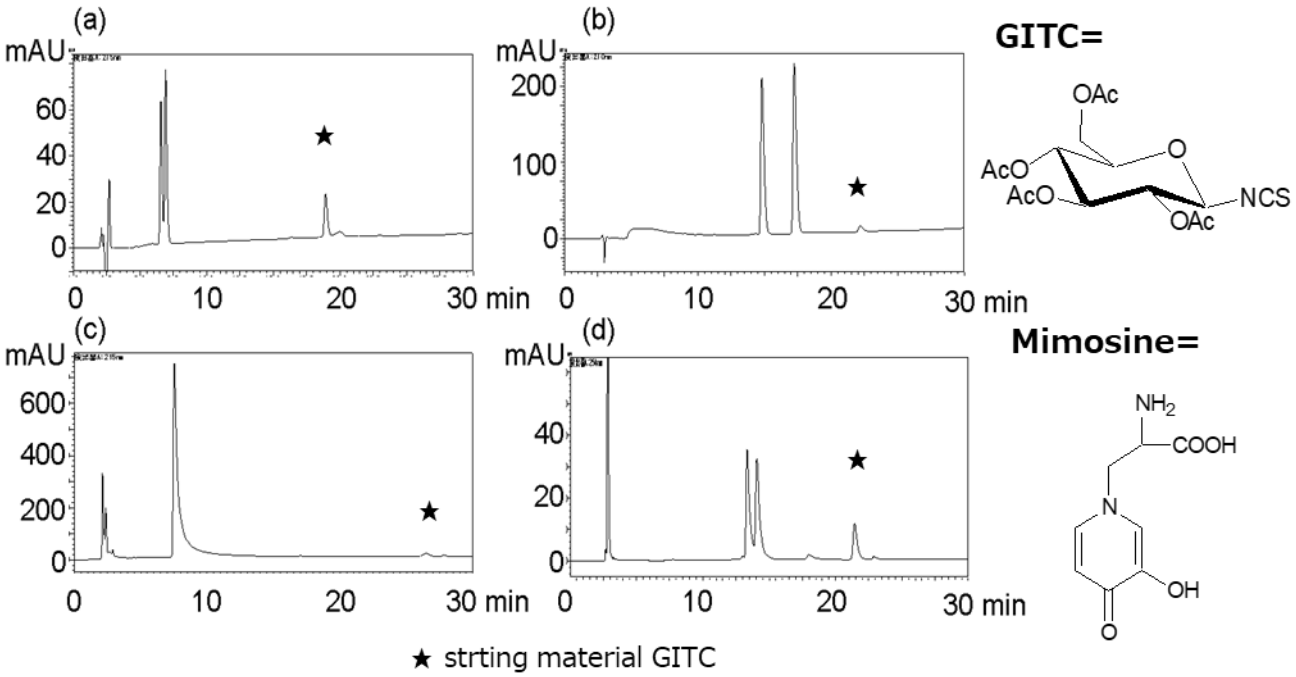
Peptide Analyses: Elution Pattern + Resolution + Recovery
→ Improved Characterization & Preparative separation by HiPep-Intrada

Peptide Science 2007, JPS, pp143, 2008 ;
Peptide Science 2008, JPS, pp 533, 2009 ;
Peptide Science 2009, JPS, pp 405, 2010;
J. Liq. Chrom & Rel. Tech, 2013, 36, 2960.

Improved chiral analysis for non proteinogenic amino acid mimosine

(a), (b) GITC-D,L-Ala; lower (c), (d): GITC-D,L-Mimosine;

Left (a), (c) = conventional ODS; Right (b), (d): HiPep-Intrada （HPLC condition ambient temp.）.



Nokihara, K, et al., *Amino Acids,* **54, 2**7-36, 2012. Preparative scale isolation, purification and derivatization of mimosine, a non-proteinogenic amino acid

Evaluation of Packing Materials Used for Preparative HPLC Purification of Peptide Derivatives

With increasing demands for pharmaceutical peptides, selection and evaluation of packing materials for HPLC, especially silica-based reverse phase, are of great interest for the pharmaceutical industries. Focusing on preparative separation of peptides and their derivatives, several model peptides have been designed. These model peptides were used for separation-tests to compare the resolution of packing materials. The results were used as a feed-back to find optimal derivatization procedures for the silica surface. In addition to efficient removal of by-products from the target peptide, recovery of desired products is also important for productivity. A GLP-1 analog, taspoglutide, consisting of 30 amino acid residues containing non-natural AAs, was chosen as a model peptide for the recovery test and for comparison of commercially available column packing materials. By-product containing peptides are often deletion peptides, which are found for sequences containing sterically hindered amino acids, cis-trans isomers of Pro-, succinimidyl Asp-, Met(O)-residues or degradation compounds caused by cleavage of Trp residues. Model peptides containing these by-products have been synthesized using conventional Fmoc-SPPS with HBTU and HOBt on an automated peptide synthesizer with low-cost Wang-resin in DMF. Cleaved peptides were characterized with a high resolution reverse phase HPLC-column, HiPep-Cadenza (3 micron ODS, 3 id. X 150 mm), using on-line ion-trap MS to identify the desired peptide and by-products.

Several test columns (2 id. X 250 mm) were prepared and, for comparison, commercial ODS was also used. Based on the above tests a novel reverse phase silica for preparative separation has been developed and designated Daisogel®SP (octadesyl silica, 10 micro meter particles, 100 and 120 angstrom porosity). The recovery was calculated by the yield after re-chromatography. Both materials gave >65% recovery and the 100 angstrom material was slightly better than the 120 angstrom product for the model peptide.

The homogeneity of the resulting purified peptides was confirmed by LCMS using the above HiPep-Cadenza to give >95% purity (calculated from peak area). These materials were packed in columns (5 id. X 500 mm) and several peptides were purified on a gram scale with flow rates of 35-50 mL/min.

Packing material (10 micron)	Yield from crude peptide	Recovery
Daisogel®SP-100-ODS-P (100A)	14.3%	68.2%
Daisogel®SP-120-ODS-RPS (120A)	11.8%	66.4%
Commercially available 100A-C18 from a leading company	11.1%	32.5%

★ Presented at the 31st European Peptide Symposium in 2010 (reprint of proceedings is available upon request)

Efficient Solid-Phase Synthesis and Purification of β-Amyloid Peptides by Improved Protocols with a Novel HPLC Column

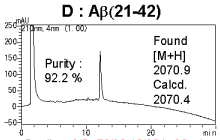
Miyazato, N., Hirata, A., Kawakami, H., Kawahira, N., Ohyama, T., and Nokihara, K. (*Correspondence:info@hipep.jp)
HiPep Laboratories, Kyoto and Okinawa, Japan (http://www.hipep.jp/)

1 Synthesis protocols

Synthesizer : PSSM-8 (Automated assemble without monitoring)
Resin : TentaGel® (Fmoc-AA TGS PHB), Strategy : PyBOP / HOBt / DIEA
Reaction Solv. Super mix (Ref : *Int.J.Peptide Res.Therapeutics* (2007) 13, 377-386.
Deprotection : 2% PIP, 2% DBU in DMF, Resin : ca. 60 mg (15 μmol)

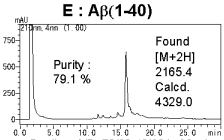
2 HPLC profiles of crude peptides by ODS columns (HiPep-Cadenza)

D : Aβ(21-42)



Gradient:A/B=70/30-10/90 in 30 min

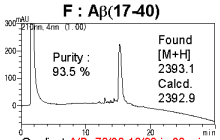
E : Aβ(1-40)



Gradient:A/B=70/30-40/60 in 30 min

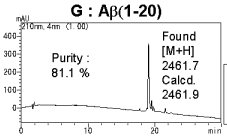
Eluent : A=0.1% TFA, B=0.1% TFA-90% ACN
Column : ϕ3.0×150 mm
Flow Rate: 0.5 mL / min
Room Temp.

F : Aβ(17-40)



Gradient:A/B=70/30-10/90 in 30 min

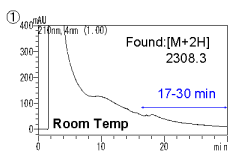
G : Aβ(1-20)



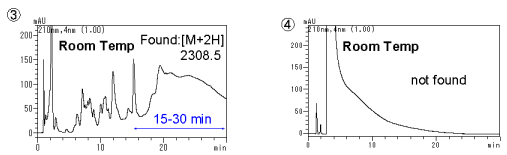
Gradient : A/B=80/20-50/50 in 30 min

A : Aβ(1-43), B : Aβ(1-42), C : Aβ(17-42)
A ~ C characterization → difficult by conventional ODS columns

3 HPLC-Profiles of crude Aβ(1-43) (mwt : calcd. 4615.1) Desired peptide was monitored by LC-MS



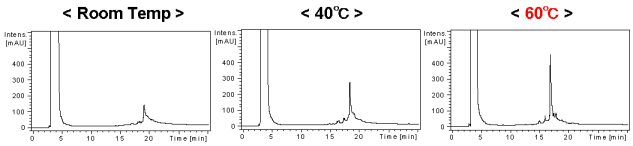
HiPep-Cadenza (ϕ3.0×150 mm)
Eluent :A=0.1% TFA, B=0.1% TFA-90% ACN, Gradient : A/B=80/20-50/50 in 30 min



HiPep-Cadenza (ϕ3.0×150 mm)
Eluent: A=NH4OH (pH 9.0), B = 90 % ACN
Gradient : A/B=90/10-30/70 in 30 min

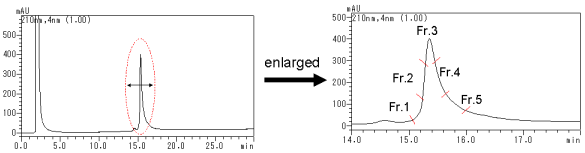
Polymer Column (ϕ4.6×300 mm)
Eluent: A=NH4OH (pH 9.0), B = 90 % ACN
Gradient : A/B=90/10-60/40 in 30 min

4 HPLC-profiles of synthetic Aβ(1-43) using a novel HiPep-Intrada



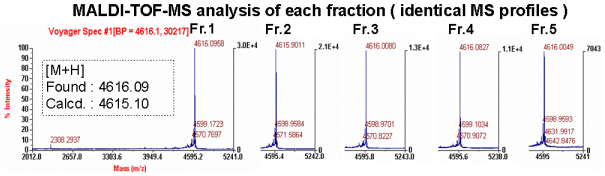
Sample : β-Amyloid(1-43)
(mwt : calcd. 4615.1, Found : [M+2H] 2307.1 respectively)
Gradient : A/B= 80/20-50/50 in 30 min
Eluent : A = 0.1 % TFA, B = 0.1 % TFA- 90 % ACN
Column : HiPep-Intrada WP-RP ϕ3.0×150 mm (P/N : HIWR03015)
Flow Rate : 0.3 mL / min
This column is commercially available info@hipep.jp

5 Further characterization of Aβ(1-43)



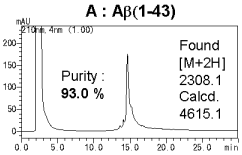
Eluent : A=0.1 % TFA, B=0.1 % TFA-90 % ACN. Gradient : A/B=80/20-20/80 in 30 min

MALDI-TOF-MS analysis of each fraction (identical MS profiles)

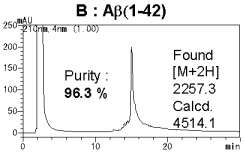


6 HPLC-profiles of synthetic Aβ related peptides by the novel HiPep-Intrada

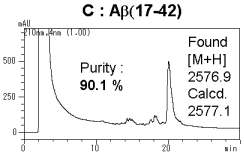
A : Aβ(1-43)



B : Aβ(1-42)



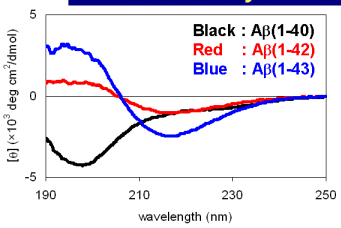
C : Aβ(17-42)



Eluent : A=0.1 % TFA H_2O,
B=0.1 % TFA-90 % ACN
Column : HiPep-Intrada WP-RP ϕ3.0×150 mm (P/N : HIWR03015)
Column Temp : 60℃,
Flow Rate : 0.5 mL/ min
Gradient : A/B= 80/20-50/50 in 30 min

Resolution was improved at 60℃ (HiPep-Intrada)
(Not efficiently characterized by the conventional ODS-column)

7 The secondary structure of Aβ peptides by CD



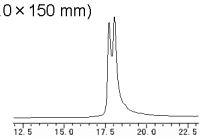
Conditions
- 10 mM sodium phosphate (pH 7.0)
- 0.1 mM peptide concentration
- Room Temperature
- Predicted based on Reed *et al.* (*Anal.Biochem.*,1997)

Secondary structure (%)

	α-helix	β-sheet	β-turn	Random coil
Aβ(1-40)	0	28.8	16.5	54.6
Aβ(1-42)	0	29.9	22.3	47.7
Aβ(1-43)	0	37.5	18.5	43.9

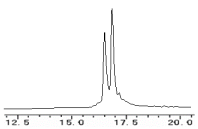
8 Separation of conformational isomers by HiPep Columns: Synthetic human Prion Protein (90-142)

HiPep-Cadenza (ODS) Column (ϕ3.0×150 mm)



Gradient : A/B=20-50% in 30 min

HiPep-Intrada (ϕ3.0×150 mm)



Gradient : A/B=10-40% in 30 min

Eluent : A=0.1% TFA, B=0.1% TFA-90% ACN, Column Temp : 60 ℃, Flow Rate : 0.5 mL / min

Separation of conformational isomers the same primary structure, the same MS data, different CD spectra. (published elsewhere)

9 Conclusion

1. The synthesis and purification of Aβ peptides could be easily performed without expensive reagent, such as pseudoproline or HATU etc. The reaction solvent system plays an important role in assembling of peptides having difficult sequences.
2. HiPep-Intrada is packed by a wide pore reversed-phase silica. (3 μm for analysis and 5 μm for preparative separation)
3. Aβ(1-43) was difficult to characterize by the conventional ODS columns, although recently developed HiPep-Intrada gave improved elution profiles. Thus Aβ(1-43) could be efficiently purified by a Prep-HiPep-Intrada (20×250 mm), which was characterized by MALDI-TOF-MS.
4. The secondary structure of the present peptides were characterized by CD spectra.
5. The present method was applied for synthesis of prion related peptides.