Selective protection and labelling of arginine/lysine side chains in HBSs of proteins

Version 3

Phuong Thao Bui[1], Quentin Nunes[1], Yong Li[1], David G.Fernig[1]

[1]University of Liverpool

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Phuong Thao Bui
University of Liverpool

ABSTRACT

Interactions between heparan sulfate proteoglycans and the partner proteins regulate many aspects of cell functions, however, their binding properties are still equivocal. Developed eight years ago was a method for selective labelling of lysine residues in the heparin binding sites of Fibroblast Growth Factors (FGFs). The labelling of arginine residues is far more challenging, due to the multiple reaction products between a dicarbonyl and a guanidino group.

EXPERIMENTAL PROCEDURES.docx

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

STEPS MATERIALS

NAME	CATALOG #	VENDOR
TOYOPEARL AF-Heparin HC-650M	0020030	
Phenylglyoxal hydrate	142433-5G	Sigma Aldrich
RapiGest SF Surfactant	View	
(P-Hydroxyphenyl)Glyoxal	24645-80-5	
Chymotrypsin, Sequencing Grade, 25ug	V1061	Promega
Iodoacetamide, 15mg	VB1010	Promega

Binding

1 AF-heparin beads (Tosoh Biosciences GmbH, Stuttgart, Germany; binding capacity of 4 mg antithrombin III/mL)

A mini affinity column was made by placing a plastic air filter as a frit at the end of a P10 pipette tip (Star Lab Ltd., Milton Keynes, UK) and then packed with 20 µL AF-heparin beads.

NOTE

Avoid bubbles inside the mini-column

The mini-column was equilibrated with 4 × 50 µL 200 mM NaCl, 0.2M $NaHCO_3$, pH 9.5 (Na-1 buffer).
The buffer was dispensed slowly into the column using a 2 mL sterile syringe.
A minimum of 10 µg FGF protein was loaded onto the column (generally, the loading capacity of FGFs to resin was estimated at 15 mg/mL). The loading was repeated 3 times to ensure the binding between protein and heparin beads. After binding, the column was washed with 200 µL (4 x 50 µL) Na-1 buffer to remove any unbound protein.

20 µl AF-heparin beads

10 µg protein

NOTE
The concentration of NaCl in Na-1 Buffer could be modified, depending on the affinity of protein to heparin

[M]0.2 Molarity (M) NaHCO3

TOYOPEARL AF-Heparin HC-650M
Catalog #: 0020030

Protection of arginine side chains

2 PGO (Merck Ltd., UK, 97%) was used in the dark, as it is light sensitive. PGO was freshly prepared in 50% (v/v) DMSO, 50% (v/v) HPLC grade water at 1 M, which was then diluted to 0.5 M and then 0.2 M with 0.2 M $NaHCO_3$, pH 9.5.
The pH was adjusted by 0.1 M NaOH to between 9.1 and 9.5 to ensure optimal reaction.
The heparin mini column was rinsed with 30 µL of the 0.2 M PGO solution to exchange buffers.

A further 30 µL PGO solution was added to the column and the bound protein was allowed to react for 60 min at room temperature in the dark.

The reaction was quenched with 5 µL 0.1% (v/v) Trifluoroacetic acid (TFA) in water so that the final concentration of TFA is 0.01% (v/v).
The mini-column was then washed with 200 µL Na-1 buffer (4 x 50 µL). Bound proteins were eluted with 2 x 20 µL Na-2 buffer (2 M NaCl, 0.2M $NaHCO_3$, pH 9.5) containing 0.1% (w/v) RapiGest SF Surfactant (Waters, UK).
The addition of surfactant was important to ensure protein recovery in this and subsequent steps, due to the increased hydrophobicity of the protein following PGO conjugation to arginine side chains.

[M]0.2 Molarity (M) PGO

60:00:00 React in the dark

Phenylglyoxal hydrate
by Sigma Aldrich
Catalog #: 142433-5G

RapiGest SF Surfactant
View

Labelling of Arginine side chain by HPG

3 The preparation of HPG was performed in the dark room as it is even more light-sensitive than PGO, following a procedure identical to that used for PGO.
The eluted protein was diluted with 400 µL 0.2M $NaHCO_3$, pH 9.5 and concentrated on a 3.5 kDa MWCO centrifugal filter to a final volume of 90~100 µL.

The reaction with HPG was performed by incubating 80 µL diluted protein with 20 µL 0.5M HPG so that the final concentration of HPG in the reaction was 0.1 M.

The pH was maintained at over 9.0. The reaction was performed for 60 min at room temperature in the dark and then was quenched with 5 µL 0.1% (v/v) TFA in water.

01:00:00 React in the dark

[M]0.1 Molarity (M) HPG

(P-Hydroxyphenyl)Glyoxal
Catalog #: 24645-80-5

Sample preparation for Mass Spectrometry

4 For labeling, protein was buffer exchanged by four cycles of dilution on 3.5 kDa-MWCO centrifugal filters with 400 µL 10-fold diluted 0.2 M $NaHCO_3$, pH 9.5 containing 0.1% (w/v) RapiGest and 3 cycles of dilution with 400 µL HPLC water containing 0.1% (w/v) RapiGest by centrifugation at 13200 rpm for 10 min.
After freezing at -80°C for 30 min, the sample was lyophilized for an hour.

EQUIPMENT
merckmillipore UFC500396
3.5 kDa-MWCO centrifugal filters

Incubation with Chymotrypsin/Trypsin

5 The freeze-dried protein was dissolved in a mixture of 80 µL 25 mM NH_4HCO_3 and 10 µL 1% (w/v) RapiGest (~0.1% w/v in final solution) and heated at 80°C for 10 min.
The mixture was quickly centrifuged at 3200 rpm for 30 seconds before 5 µL 50 mM DTT was added (5mM final concentration) and incubated for 15 min at 56°C.
After cooling the sample to room temperature, proteins were carbamidomethylated with 5 µL 0.1M iodoacetamide (freshly made) for 30 min in the dark.
Proteins were then digested overnight with chymotrypsin (Promega Ltd., UK) at a ratio of 1:100.

Chymotrypsin, Sequencing Grade, 25ug
by Promega
Catalog #: V1061

17:00:00 h

Iodoacetamide, 15mg
by Promega
Catalog #: VB1010

00:30:00 min - in the dark

Mass spectrometry for the identification of peptides

6 Peptides were concentrated by rotary evaporation to a final volume of 10 µL and desalted using C18 Zip-Tips(Millipore). C18 Zip Tips were first pre-wetted with 2 x 10 µL 100 % (v/v) acetonitrile and then pre-equilibrated with 2 x 10 µL 0.1% (w/v) TFA in water. The peptides were loaded on the Zip Tip, the loading was repeated 7 to 8 times to ensure binding. The Zip Tip was washed with 10 µL 0.1% (w/v) TFA. Finally, the peptides were eluted with 2 µL of 5mg/mL α-cyano-4-hydroxycinnamic acid (CHCA, > 99% purity, Sigma) in 50:50 acetonitrile/water + 1%TFA, straight onto a 96 spot MALDI (matrix-assisted laser desorption/ionisation) target plate.

Analyses were performed on the instrument Synapt G2-Si (Waters, Manchester, UK) with MALDI source equipped with a frequency tripled Nd:YAG UV laser (λ = 355 nm), operating at 1 kHz. The spectrum acquisition time was 120 seconds, with 1 second scan rates, laser energy of 150 Au. The MS spectra were extracted by MASSLYNX v.4.1 (Waters, Manchester, UK) with the spectrum range from 500 Da to 4000 Da. The spectra were then processed using automatic peak detection including background subtraction.