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Identification of *N*-homocysteinylation sites in plasma proteins

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Abstract A protocol for the identification of *N*-homocysteinylation sites in plasma proteins is described. Human plasma or purified fibrinogen is subjected to trypsin digestion and analysis of *N*-Hcy-peptides by liquid chromatography/mass spectroscopy (LC/MS). Human fibrinogen is isolated from the plasma by the glycine precipitation method. Identification of *N*-Hcy-Lys-peptides in tryptic digests of in vivo-derived samples is facilitated by the use of *N*-Hcy-albumin and *N*-Hcy-fibrinogen synthesized in vitro from commercially available human proteins. This protocol allows identification of *N*-homocysteinylation sites at Lys4, Lys12, Lys137, and Lys525 in albumin directly in trypsin-digested human serum samples. *N*-Hcy-Lys562, *N*-Hcy-Lys344, and *N*-Hcy-Lys385 were identified in human fibrinogen from patients with cystathionine β-synthase deficiency. The protocol can be completed in 4 days.

Keywords Homocysteine thiolactone · Human albumin · Human fibrinogen · *N*-homocysteinylation · Mass spectrometry

Introduction

Homocysteine (Hcy) is an intermediate metabolite that arises from the metabolism of the essential dietary protein amino acid methionine (Met). Hcy levels are regulated by remethylation to Met, catalyzed by Met synthase and betaine-Hcy methyltransferase, as well as by transsulfuration to cysteine, the first step of which is catalyzed by cystathionine β-synthase (CBS) (Mudd et al. 2001). Met and Cys are two canonical coded amino acids that are incorporated by the ribosomal biosynthetic apparatus into polypeptide chains of proteins. In contrast, Hcy is a non-coded amino acid that normally does not participate in ribosomal protein biosynthesis. However, studies beginning in the end of 1990s have identified mechanisms by which Hcy becomes a constituent of proteins (Jakubowski 2012, 2013). In one of those mechanisms, Hcy is first metabolized to the thioester Hcy-thiolactone in an error-editing reaction in protein biosynthesis when Hcy is erroneously selected in place of Met by methionyl-tRNA synthetase (Jakubowski 2011, 2012). Hcy-thiolactone then forms isopeptide bonds with protein lysine residues (Fig. 1) (Jakubowski 1997, 2000; Jakubowski et al. 2000) in a process called *N*-homocysteinylation. The incorporation of Hcy into isopeptide bonds alters protein's structure/function (Jakubowski 1999; Glowacki and Jakubowski 2004), is autoimmunogenic (Undas et al. 2004), and

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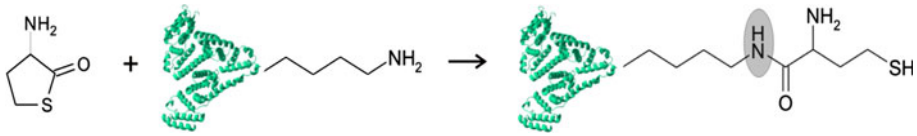

Fig. 1 *N*-homocysteinylation of a protein lysine residue by Hcy-thiolactone (Jakubowski 1997, 2013)

contributes to Hcy toxicity (Jakubowski 2004, 2007, 2013; Perla-Kajan et al. 2008). Protein *N*-homocysteinylation occurs efficiently at nanomolar Hcy-thiolactone concentrations present in human plasma and increases linearly with increasing Hcy-thiolactone levels (Jakubowski 1999; Chwatko et al. 2007).

The major human plasma proteins which undergo *N*-homocysteinylation in vivo are fibrinogen and albumin (Jakubowski 2002; Glowacki and Jakubowski 2004; Jakubowski et al. 2008). *N*-homocysteinylation of lysine residues results in a loss of positive charge and a gain of a thiol group in a protein molecule. As a consequence, modified *N*-Hcy-proteins become more susceptible to oxidative damage, aggregation (Jakubowski 1999; Glowacki and Jakubowski 2004), and amyloidal transformation (Paoli et al. 2010). Approximately, 70 % of circulating Hcy is *N*-linked to blood proteins, mostly albumin and hemoglobin, and the other 30 % include disulfide bound and free Hcy forms and Hcy-thiolactone (Jakubowski 2002).

Elevated plasma Hcy (>10 μM) is associated with an increased risk of myocardial infarction, stroke, thromboembolism, neurodegenerative diseases, and pregnancy complications (Maron and Loscalzo 2009). Severe hyperhomocysteinemia (Hcy >100 μM) observed in human CBS deficiency has a worldwide incidence of 1:3,00,000 and is associated with mental retardation, ectopia lentis, osteoporosis, and vascular complications (thromboembolism), which are the major causes of morbidity and mortality in untreated CBS-deficient patients (Mudd et al. 2001). Hcy-thiolactone and *N*-Hcy-protein levels are elevated in CBS deficiency, both in humans and mice (Jakubowski 1997; Chwatko et al. 2007; Jakubowski et al. 2008, 2009), and contribute to the pathology of hyperhomocysteinemia (Jakubowski 2013).

The quantitative analysis of *N*-Hcy-protein levels requires acid hydrolysis, during which protein *N*-linked Hcy is converted to Hcy-thiolactone, which is then detected and quantified by high-performance liquid chromatography (HPLC) (Jakubowski 2002, 2008). Immunological methods using rabbit polyclonal anti-*N*-Hcy-protein antibodies are also available for the detection of *N*-Hcy-protein (Yang et al. 2006; Perla-Kajan et al. 2008). Chemical tagging methods, using fluorescent or biotin aldehyde tags, for the analysis of *N*-Hcy-proteins are being explored (Zang et al. 2009).

Identification of lysine residues in proteins that are targeted for *N*-homocysteinylation in vivo is important in understanding their role in human health and disease (Jakubowski 2013). The protocol described herein facilitates identification of the site-specific *N*-homocysteinylation and is based on a liquid chromatography/mass spectrometry (LC/MS) method that monitors specific *N*-Hcy-Lys-peptides generated by the digestion of human plasma protein with trypsin (Sikora et al. 2010; Marczak et al. 2011). Using in vitro-prepared samples of plasma *N*-Hcy-proteins as standards allows unambiguous identification of specific sites of *N*-homocysteinylation in albumin and fibrinogen. This protocol enables identification of *N*-homocysteinylation sites at Lys4, Lys12, Lys137, and Lys525 in albumin directly in trypsin-digested human serum samples (Sikora et al. 2010; Marczak et al. 2011). Furthermore, this protocol is also suitable for the detection of specific *N*-Hcy-Lys residues in human fibrinogen isolated from CBS-deficient patients: *N*-Hcy-α-Lys562, *N*-Hcy-β-Lys344, and *N*-Hcy-γ-Lys385 (Sikora et al. 2012).

Materials

Reagents

Human serum albumin (Sigma; http://www.sigmaaldrich.com; cat. no. A9511).
Human fibrinogen (Sigma; http://www.sigmaaldrich.com; cat. no. F3879).
Human plasma from the Danish (Maclean et al. 2002), Dutch (Jakubowski et al. 2008), and Polish CBS-deficient patients (Orendae et al. 2004), and unaffected subjects (use of the human plasma samples has been approved by the Local Ethical Committee, Poznań).
Glycine (Sigma; http://www.sigmaaldrich.com; cat.no. 50046).
Dithiotreitol (DTT) (Sigma; http://www.sigmaaldrich.com; cat. no. 403817).
Iodoacetamide (IAA) (Sigma; http://www.sigmaaldrich.com; cat. no. I6125).
Ammonium bicarbonate for LC–MS (Sigma; http://www.sigmaaldrich.com; cat. no. 40867).
Sequencing grade modified trypsin (Promega, cat. no. V5111).
Sodium phosphate monobasic monohydrate (Sigma; http://www.sigmaaldrich.com; cat. no. S9638).
Sodium phosphate dibasic anhydrous (Sigma; http://www.sigmaaldrich.com; cat. no. 71640).

Trifluoroacetic acid (TFA) (Sigma; http://www.sigmaaldrich.com; cat. no. 302031).
L-Homocysteine thiolactone hydrochloride (Sigma; http://www.sigmaaldrich.com; cat. no. 53527).
Ethylenediaminetetraacetic acid (0.5 M EDTA, pH 8.0) (http://www.bioshopcanada.com; cat. no. EDT333.100).
PBS tablets (http://www.bioshopcanada.com; cat. no. PBS404.200).
Urea (Sigma; http://www.sigmaaldrich.com; cat. no. UO631).

Equipment

pH meter Basic 20 (www.labindex.pl).
Dry bath (www. http://europe.labnetinternational.com; cat. no. D1100).
Centrifuge (Eppendorf, cat. no. 5424R).
Nano LC system Proxeon Easy NanoLC (Bruker Daltonics; http://www.bruker.com).
micrOTOF-q tandem mass spectrometer (Bruker Daltonics; http://www.bruker.com).
MALDI-ToF mass spectrometer (Bruker Daltonics; http://www.bruker.com).
Mascot server 2.3.0 software (Matrix Science Ltd.; http://www.matrixscience.com).

Reagent setup

0.1 M sodium phosphate buffer, pH 7.4 3.1 g NaH_2PO_4, 10.9 g Na_2HPO_4 (anhydrous), deionized H_2O to 1 L. The pH of the final solution will be 7.4; check with a pH meter. Can be stored for up to 1 month at 4 °C.

L-Hcy-thiolactone stock solution (0.1 M) 15.4 mg L-Hcy-thiolactone·HCl, deionized H_2O to 0.1 mL in a plastic Eppendorf tube. Can be stored indefinitely at −20 °C.

CRITICAL Although concentrated aqueous L-Hcy-thiolactone·HCl solutions are stable, diluted solutions may decompose during storage. L-Hcy-thiolactone·HCl in buffered solutions decomposes slowly ($t_{0.5}$ = 30 h at pH 7.4, 37 °C). The decomposition is accelerated in the presence of proteins, amines, or aldehydes (Jakubowski 2004, 2007, 2013; Jakubowski and Glowacki 2011).

Dithiothreitol stock solution (0.1 M) 15 mg DTT, deionized H_2O to 1 mL in a plastic Eppendorf tube.

CRITICAL Dithiothreitol solution should be used fresh.

Iodoacetamide stock solution (0.1 M) 18 mg IAA, deionized H_2O to 1 mL in a plastic Eppendorf tube.

CRITICAL Iodoacetamide solution should be used fresh, as it is photosensitive.

Trypsin stock solution (0.2 μg/μL) Dissolve the contents in 0.1 mL of trypsin buffer provided by the manufacturer. Make 20 μL aliquots and store in −20 °C.

CRITICAL Trypsin solution should be always used fresh; do not freeze it twice.

Ammonium bicarbonate stock solution (0.05 M) 4 mg Ammonium bicarbonate, deionized H_2O to 1 mL in a plastic Eppendorf tube.

CRITICAL Ammonium bicarbonate solution should be used fresh.

Glycine stock solution (3.5 M) 0.52 g Glycine, deionized H_2O to 2 mL in a plastic Eppendorf tube.

Sodium chloride stock solution (1 M) 0.584 g NaCl, deionized H_2O to 10 mL in a plastic test tube.

Washing buffer for fibrinogen isolated from human plasma Mix 0.3 ml 3.5 M glycine, 0.05 mL 1 M NaCl, 0.05 ml of 10 mM EDTA, and 0.1 mL 0.1 M sodium phosphate buffer, pH 7.4.

Equipment setup

pH meter calibration

Turn on the pH meter. Select calibration mode from 7 to 10. Immerse the electrode into the certified buffer solution (pH 7). Wait until the reading stops blinking and a beeping sound is heard. Remove the buffer solution. Rinse the electrode with deionized water. Immerse the electrode into the certified buffer solution (pH 10). Wait until the reading stops blinking and a beeping sound is heard. Remove the buffer solution. Rinse the electrode with deionized water. Remove the excess water with a paper tissue. Timing is 10 min.

Mass spectrometry

Separation of peptides derived from trypsin digestion was carried out using LC/MS system consisting of Proxeon Easy nano LC chromatograph directly connected to Bruker Daltonics (Bremen, Germany) micrOTOF-q mass spectrometer. HPLC was equipped with 2 cm-long C18 trap column and 10 cm C18 analytical column; 300 nL/min flow was used and peptides were separated using 5–60 % acetonitrile gradient in 40 min.

The parameters of Q-ToF mass spectrometer were as follows: drying gas flow in the ion source was set up to 4 L/h and nebulizer gas pressure to 0.4 bar (both were N2), the temperature of ion source was 140 °C, voltage was set to 4.5 kV, and collision energy of CID for double charged ions ranged between 25 and 50 eV and was dependent on ion *m*/*z* value. The instrument was operated at a resolution higher than 15,000 (FWHM, full width at half maximum) under micrOTOF-Q Control ver. 2.3 and data were analyzed using DataAnalysis ver. 4 package delivered by Bruker Daltonics. The data were acquired as a continuous cycle of MS and MS/MS acquisitions of the five most intense peaks.

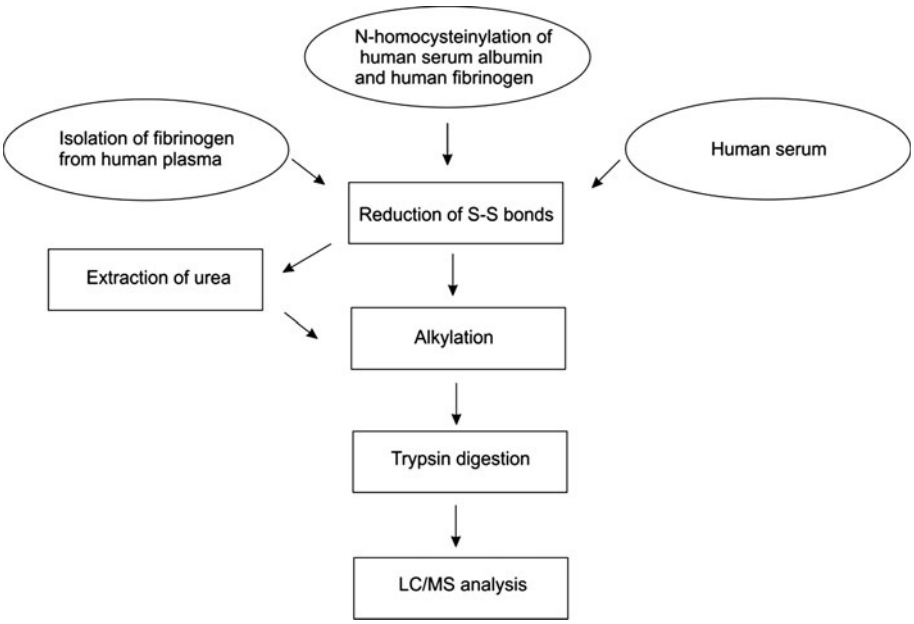

Fig. 2 Work flow diagram of the procedures described in this protocol

Procedure

A work flow diagram of the protocol is shown in Fig. 2.

Preparation of N-Hcy-fibrinogen and N-Hcy-albumin in vitro ● ***TIMING*** *16 h*

1. Add 10 µL 20 µg/µL human fibrinogen or albumin solution to 8 µL 0.1 M sodium phosphate buffer, pH 7.4; then add 2 µL 0.1 M L-Hcy-thiolactone and 0.2 µL 20 mM EDTA.
2. To the second tube add 10 µL 20 µg/µL human fibrinogen or albumin solution to 10 µL 0.1 M sodium phosphate buffer, pH 7.4 and 0.2 µL 20 mM EDTA, as a control.
3. Incubate all tubes at 37 °C overnight.

Isolation of fibrinogen from human plasma ● ***TIMING*** *8 h*

4. Add 33 mg glycine to 0.2 mL of cold plasma to precipitate fibrinogen (Mosesson and Sherry 1966; Jakubowski et al. 2008).
5. Keep the sample on ice, occasionally mixing, until glycine is dissolved.
6. Collect precipitated fibrinogen by centrifugation (18,000×*g*, 4 °C, 20 min).
7. Wash the fibrinogen pellet twice with the washing buffer by discarding the supernatant and replacing with 0.05 mL of washing buffer.
8. Dissolve the pellet in phosphate-buffered saline.

Protein reduction–fibrinogen ● ***TIMING*** *16 h*

9. Distribute samples of in vitro-prepared *N*-Hcy-fibrinogen (3 µL), fibrinogen isolated from human plasma (5 µL), and control fibrinogen (3 µL) to the separate 0.5 mL Eppendorf tubes.
10. Add 1 µL 1 M DTT and 30 µL 8 M urea in sodium phosphate buffer, pH 7.4, to each tube.
11. Incubate all tubes overnight at 37 °C.

Extraction on C4 columns ● ***TIMING*** *1 h*

12. Activate ZipTip C4 solid phase extraction microcolumns by rinsing three times with 10 µL 50 % acetonitrile, 0.1 % TFA, followed by three times with 10 µL 0.1 % TFA. **! CAUTION** acetonitrile is toxic. When handling, wear gloves and use a pipetting aid.
13. Aspirate each sample ten times into separate tip to bind proteins to the C4 resin.
14. Wash each tip three times with 0.1 % TFA.
15. Elute bound proteins with 10 µL 50 % acetonitrile.
16. Dry the eluted proteins in a vacuum centrifuge.
17. Reconstitute in 10 µL 50 mM ammonium bicarbonate.

Protein reduction–albumin ● ***TIMING*** *10 min*

18. Add 5 µL human plasma to 45 µL deionized water to prepare 10x diluted plasma.

19. Distribute samples of diluted plasma (10 μL), in vitro-prepared *N*-Hcy-albumin (5 μL), and control albumin (5 μL) to the separate 0.5 mL Eppendorf tubes.
20. Add 2 μL 0.1 M DTT and 23 μL 50 mM ammonium bicarbonate.
21. Incubate the samples at 95 °C for 5 min.

Carbamidomethylation of protein thiol groups ● ***TIMING*** *0.5 h*

22. Add 8 μL 1 M IAA to each tube and incubate for 30 min at 37 °C in darkness.

CRITICAL STEP: Keep the samples in darkness, as IAA is light sensitive.

Tryptic digestion ● **TIMING** 16 h.

23. Add 2 μL 0.2 μg/μL trypsin to each tube and incubate at 37 °C overnight.

Mass spectrometry and data analysis ● ***TIMING*** *2 days*

24. Load 10 μL of tryptic digest on nano-LC–MS/MS and generate MS/MS spectra as described in the equipment setup and in previous publications (Sikora et al. 2010; Marczak et al. 2011).
25. Interpret MS/MS spectra and generate peak lists using Data Analysis 4.0.
26. Use Mascot 2.3 to search against the Swissprot database limited to human species for peptide identification. Set searching parameters as follows: select enzyme as trypsin with three maximum missing cleavage sites, use mass tolerance of 0.2 Da, use 0.2 Da for MS/MS tolerance, set fixed modification as carbamidomethyl (C) and variable modification as methionine oxidation.
27. Because *N*-Hcy-Lys is not present in the primary version of the search engine, we introduced this modification into the MASCOT database. This was done by entering "Configuration Editor" and clicking on "Modifications" option. Next, "Add new modification" was chosen, C(6) H(10) N(2) O(2) S(1) (for *S*-carbamidomethylated *N*-linked Hcy) was introduced in the composition window, and Lys (K) as modification site under the specificity tab. Mass increase due to Lys modification by *S*-carbamidomethylated *N*-linked Hcy is 174 Da. The Swissprot database was used for the search.

● ***TIMING***

Step 1–3 **Preparation of *N*-Hcy-fibrinogen and *N*-Hcy-albumin in vitro** 16 h
Step 4–8 **Isolation of fibrinogen from human plasma** 8 h
Step 9–11 **Protein reduction—fibrinogen** 16 h
Step 12–17 **Extraction on C4 columns** 1 h
Step 18–21 **Protein reduction—albumin** 10 min
Step 22–23 **Alkylation of protein thiol groups** 30 min
Step 24–27 **Mass spectrometry and data analysis** 2 days

? TROUBLE SHOOTING

Problem	Possible reasons	Solution
Glycine dissolves very slowly	Glycine is not pure	Use glycine with higher purity. Tap the tube from time to time.
Digestion of proteins is not complete	Activity of trypsin is low	To maintain maximum trypsin activity, limit the number of freeze–thaw cycles to five or dispense into single-use aliquots after re-suspending.
	The pH is too low	Always use fresh solution of ammonium bicarbonate (the pH should be ~8)
Keratins listed in identification report (MASCOT)	Sample was contaminated with keratins	Use gloves and work under hood to avoid contaminations.
N-Hcy Lys identified on a C-terminal lysine residue	Not a correct assignment	Trypsin does not cut after modified lysine residues, thus *N*-Hcy-Lys residue cannot be present at the C-terminus (see Figs. 3 and 5; Tables 1 and 2).
Do not have access to in-house MASCOT server		Use other search engines, use FindMod (SwissProt) for MS data obtained on MALDI-ToF systems.

Anticipated results

Serum albumin is the major target for *N*-homocysteinylation in the human blood (Jakubowski 2002; Glowacki and Jakubowski 2004). While Lys525 is a predominant site for *N*-homocysteinylation in human serum albumin (Glowacki and Jakubowski 2004), three other lysine residues—Lys4, Lys12, and Lys137—are also susceptible to *N*-homocysteinylation in vitro and in vivo (Table 1; Fig. 3), as demonstrated using the present protocol (Sikora et al. 2010; Marczak et al. 2011; Jakubowski 2013). Fibrinogen is also

Table 1 Tryptic peptides from *N*-Hcy-albumin identified as carrying *N*-linked Hcy

Peptide no.[a]	*m/z* meas.	Mr calc.	Sequence[b, c]	Modifications	Range	*N*-Hcy site[c]
1	1,323.6	1,322.6	.DAH***K****SEVAHR.F	*N*-Hcy:4	1–10	**Lys4**[c]
2	1,400.6	1,399.6	R.F***K****DLGEENFK.A	*N*-Hcy: 2	11–20	**Lys12**[c]
3	1,229.6	1,228.6	K.***K****YLYEIAR.R	*N*-Hcy: 1	137–144	**Lys137**[c]
4	2,073.1	2,072.0	R.HPYFYAPELLFFA*K**R.Y	*N*-Hcy: 14	146–160	Lys159
5	1,370.6	1,369.6	K.CASLQ*K**FGER.A	*N*-Hcy: 6; Carbamidomethyl: 1	201–210	Lys205
6	1,193.6	1,192.6	R.AF*K**AWAVAR.L	*N*-Hcy: 3	211–219	Lys212
7	1,302.8	1,301.7	K.***K****QTALVELVK.H	*N*-Hcy: 1	525–534	**Lys525**[c]

[a] Peptides 1–7 are present in tryptic digests of *N*-Hcy-albumin prepared in vitro. Peptides 1, 2, 3, and 7 are present in tryptic digests of serum protein in vivo from CBS-deficient patients, while peptide 7 is also present in tryptic digests of serum protein from unaffected subjects

[b] *K** denotes lysine residues carrying *N*-linked Hcy

[c] Lysine residues that carry *N*-linked Hcy in vivo are indicated in bold

targeted for *N*-homocysteinylation in human plasma in vivo (Jakubowski 2002; Jakubowski et al. 2008). The present protocol allows identification of specific lysine residues in human fibrinogen that are *N*-homocysteinylated both in vitro and in vivo (Sikora et al. 2012; Jakubowski 2013). Using this protocol, we identified 17 lysine residues in human fibrinogen that are susceptible to *N*-homocysteinylation in vitro (Table 2; Fig. 4). *N*-homocysteinylation at three of those lysine residues is detected in human fibrinogen in vivo (Fig. 5). Other investigators identified 12 *N*-Hcy-peptides in human *N*-Hcy-fibrinogen prepared in vitro (Sauls et al. 2006).

Changes in molecular mass of proteins modified with Hcy-thiolactone in vitro are monitored using MALDI-ToF and ESI/MS systems. The difference between the mass of intact protein and *N*-Hcy-protein containing one *N*-linked Hcy is 174 Da (elemental composition of molecule substituted on amino group of lysine is $C_6H_{10}O_2N_2S$, mass of a single Hcy molecule is 117 Da, and iodoacetamide treatment increases its mass by 57 Da). To identify sites of specific *N*-homocysteinylation in human albumin or fibrinogen in vivo, we analyzed tryptic digests of human serum or purified fibrinogen obtained from plasma of CBS-deficient patients using nano-LC/ESI–MS/MS.

Four albumin peptides, containing *N*-Hcy-Lys525, *N*-Hcy-Lys137, *N*-Hcy-Lys12, and *N*-Hcy-Lys4, first identified in samples of in vitro *N*-homocysteinylated albumin (Marczak et al. 2011) were also found in trypsin-digested human serum samples (i.e., in vivo) (Table 1). Extracted ion chromatograms for peptides containing *N*-Hcy-Lys4, 12, 137, and 525 are shown in Fig. 3a. The structure of these peptides is confirmed by MS/MS fragmentation, as illustrated for the *N*-Hcy-Lys525-peptide in Fig. 3b. The *N*-Hcy-Lys-525-peptide is present in 43 out of 44 analyzed serum samples, including those which have the lowest tHcy concentration (9.9 μM) (Marczak et al. 2011). There is a significant correlation between the albumin *N*-Hcy-Lys525-peptide and plasma total Hcy ($r = 0.49$, $p < 0.001$) (Sikora et al. 2010). Two other albumin peptides, containing *N*-Hcy-Lys137 and *N*-Hcy-Lys212, are present in serum samples in which tHcy concentration is higher, at least 34.9 ± 11.0 μM and 131 ± 21 μM, respectively (Marczak et al. 2011).

In human fibrinogen incubated with Hcy-thiolactone in vitro, 17 lysine residues susceptible to *N*-homocysteinylation are identified (Sikora et al. 2012): Lys52, Lys70, Lys81, Lys138, and Lys562 in α-chain; Lys181, Lys217, Lys 298, Lys344, and Lys396 in β-chain; Lys85, Lys95, Lys170, Lys266, Lys273, Lys373, and Lys385 in γ-chain. The locations of these residues in fibrinogen peptides are indicated in Table 2, while relative amounts of *N*-Hcy-Lys-peptides containing these lysine residues are shown in Fig. 4. Three of these lysine residues, one in each subunit α-Lys562, β-Lys344, γ-Lys385, are *N*-homocysteinylated in vivo and present in fibrinogen isolated from plasma of CBS-deficient patients. Extracted ion chromatograms for the three fibrinogen *N*-Hcy-Lys-peptides are shown in Fig. 5a. The structures of these peptides are confirmed by fragmentation analysis, illustrated for the *N*-Hcy-α-Lys562-peptide in Fig. 5b.

The α-subunit Lys562 site of *N*-homocysteinylation is located in an unstructured region of the αC domain known to be involved in tPA and plasminogen binding (α392–610), which can explain the abnormal characteristics of clots formed from *N*-Hcy-fibrinogen (Sauls et al. 2006). *N*-Hcy-Lys562 is close to the sites of two mutations Ser532->Cys and Arg554-Cys that are associated with thrombosis (Koopman et al. 1993; Marchi et al. 2000). Thus, it is likely that *N*-Hcy-Lys562 contributes to the prothrombotic properties of *N*-Hcy-fibrinogen in CBS-deficient patients (Sikora et al. 2012; Jakubowski 2013).

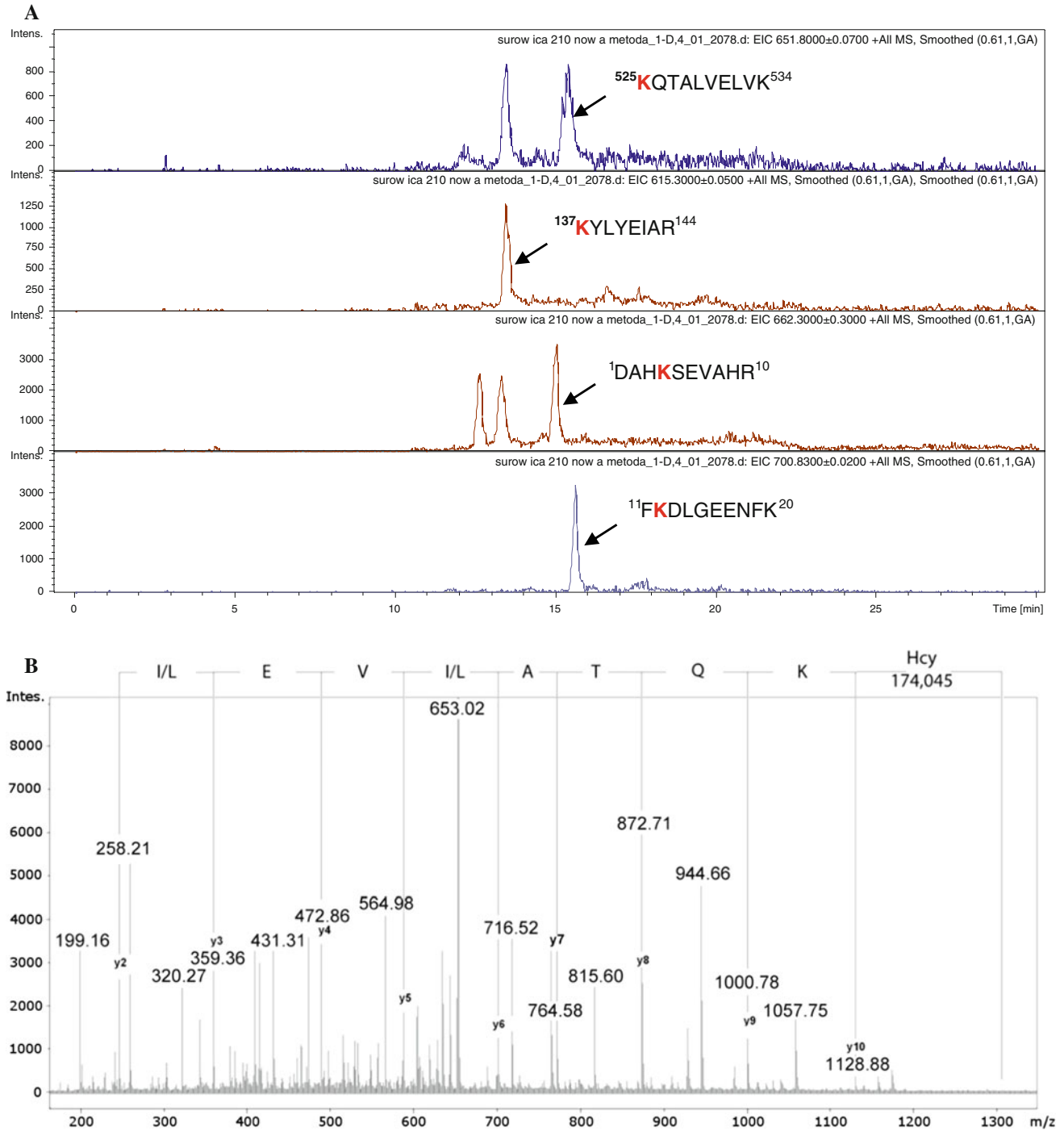

Fig. 3 **a** LC–ESI/MS chromatograms of selected ions for *N*-Hcy-Lys-peptides of albumin detected in human serum from a CBS-deficient patient (with plasma Hcy,=27 μM): ^{525}(*N*-Hcy-K)QTALVELVK^{534}, ^{137}(*N*-Hcy-K)YLYEIAR^{144}, ^{1}DAH(*N*-Hcy-K)SEVAHR^{10}, ^{11}F(*N*-Hcy-K)DLGEENFK^{20}. **b** Q-ToF fragmentation spectra of *N*-Hcy-Lys525-peptide of albumin

Hcy-thiolactone is known to modify only lysine residues in proteins (Jakubowski 1999). *N*-Hcy-Lys residues in each of the identified tryptic peptides are either internal or N-terminal, but never occur at the C-terminus; this is consistent with the specificity of trypsin, which does not cut after modified lysine residues.

This protocol allows identification of *N*-homocysteinylation sites in serum albumin directly in serum samples and in fibrinogen after one-step purification by glycine precipitation. The *N*-homocysteinylation status of specific lysine residues in these proteins could be an indicator of the efficacy of the treatment of hyperhomocysteinemia.

Table 2 Tryptic peptides from *N*-Hcy-fibrinogen identified as carrying *N*-linked Hcy

Peptide no.[a]	*m/z* measured	Mr calculated	Sequence[b]	Modifications	Range[c]	*N*-Hcy site[c, d]
Fibrinogen alpha chain						
1	1,443.7	1,442.7	K.L*K**NSLFEYQK.N	*N*-Hcy: 2	69–78	Lys70
2	1,649.9	1,648.9	K.VQHIQLLQ*K**NVR.A	*N*-Hcy: 9	130–141	Lys138
3	1,953.9	1,952.9	R.M*K**GLIDEVNQDFTNR.I	*N*-Hcy: 2	51–64	Lys52
4	2,003.9	2,002.9	K.SSSYS***K****QFTSSTSYNR.G	*N*-Hcy: 6	557–572	**Lys562**[c]
5	2,161.0	2,159.0	K.NN*K**DSHSLTTNIMEILR.G	*N*-Hcy: 3	79–95	Lys81
Fibrinogen beta chain						
6	1,344.7	1,343.7	K.YQISVN***K****YR.G	*N*-Hcy: 7	338–346	**Lys344**[c]
7	1,916.8	1,915.8	K.QCS*K**EDGGGWWYNR.C	*N*-Hcy: 4; Carbamidomethyl: 2	393–406	Lys 396
8	2,130.9	2,130.0	K.IQ*K**LESDVSAQMEYCR.T	*N*-Hcy: 3; Carbamidomethyl:15	179–194	Lys 181
9	2,560.2	2,558.2	R.KGGETSEMYLIQPDSSV*K**PYR.V	*N*-Hcy: 1	217–237	Lys 217
10	2,659.2	2,657.2	K.NYCGLPGEYWLGND*K**ISQLTR.M	*N*-Hcy: 15; Carbamidomethyl: 3	284–304	Lys 298
Fibrinogen gamma chain						
11	1,394.7	1,393.7	K.TTM***K****IIPFNR.L	*N*-Hcy: 4	382–390	**Lys385**[c]
12	1,467.8	1,466.8	K.QSGLYFI*K**PLK.A	*N*-Hcy: 8	163–173	Lys170
13	2,324.0	2,323.1	R.TSTADYAMF*K**VGPEADKYR.L	*N*-Hcy: 10	257–275	Lys266
14	2,326.1	2,324.1	K.ASTPNGYDNGIIWATW*K**TR.W	*N*-Hcy: 17	357–375	Lys373
15	2,498.1	2,497.1	R.TSTADYAMF*K**VGPEAD*K**YR.L	*N*-Hcy: 10, 17	257–275	Lys273
16	2,540.2	2,539.2	K.MLEEIM*K**YEASILTHDSSIR.Y	*N*-Hcy: 7	89–108	Lys95
17	2,937.4	2,936.4	K.AIQLTYNPDESSKPNMIDAATL*K**SR.K	*N*-Hcy: 23	63–87	Lys85

[a] Peptides 1–17 are present in tryptic digests of *N*-Hcy-fibrinogen prepared in vitro. Peptides 4, 6, and 11 are present in tryptic digests of fibrinogen isolated in vivo from CBS-deficient patients

[b] ***K**** denotes lysine residues carrying *N*-linked Hcy

[c] Residue numbering does not include signaling sequences and reflects the residue numbers in the mature chains

[d] Lysine residues that carry *N*-linked Hcy in vivo are indicated in bold

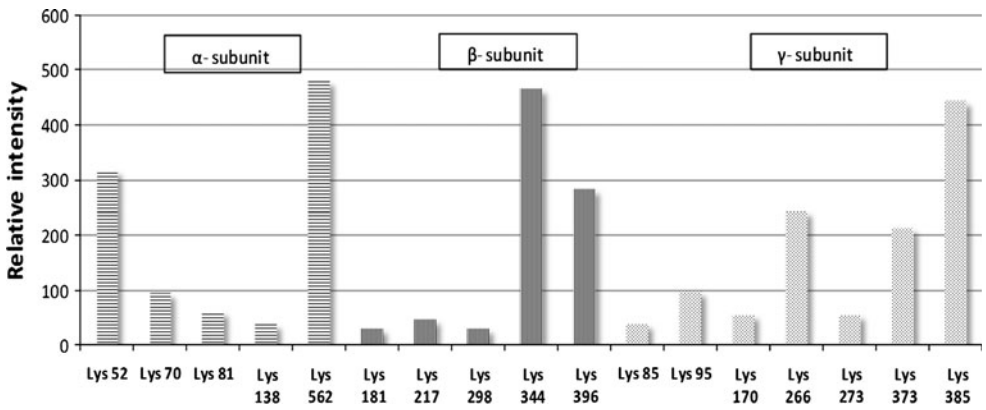

Fig. 4 Relative amounts of specific *N*-Hcy-Lys-peptides obtained by digestion with trypsin of human *N*-Hcy-fibrinogen prepared in vitro and identified by LC–ESI–MS analysis (*horizontal stripes* α-subunit; *vertical stripes* β-subunit; *dots* γ-subunit). Positions of *N*-homocysteinylated lysine residues in the primary structure of fibrinogen subunits are indicated by numbers on the abscissa; relative signal intensity for each *N*-Hcy-Lys-peptide containing indicated lysine residue is shown on the ordinate

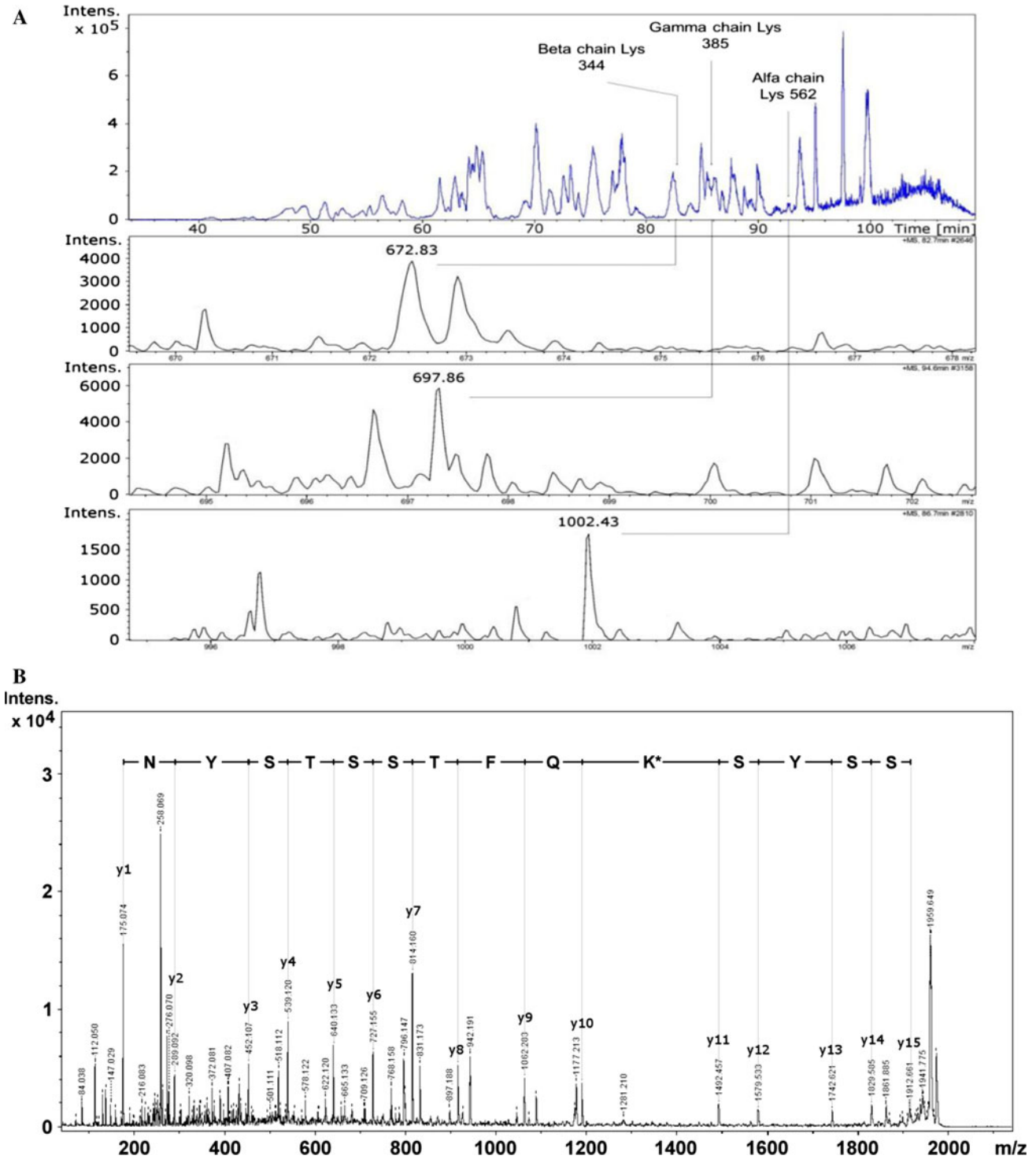

Fig. 5 **a** LC MS/MS analysis of tryptic digest of fibrinogen purified from a CBS-deficient patient (with plasma Hcy = 147 μM) : top panel, LC profile of peptides (BPC); three bottom panels show fragments of spectra for *N*-Hcy-Lys-peptides (double charged ions $[M+2H]^{2+}$) containing β-Lys344 (*m*/*z* 672.83), α-Lys562 (*m*/*z* 1,002.43), and γ-Lys 385 (*m*/*z* 697.86). **b** MALDI-ToF/ToF fragmentation spectrum of the fibrinogen *N*-Hcy-αLys562-peptide consistent with its structure ^{557}SSSYS(*N*-Hcy-Lys)QFTSSTSYNR^{572}. K* denotes *N*-Hcy-Lys modification

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Conflict of interest The authors declare that they have no competing financial interests.

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