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# Aminoacylase 3 binds to and cleaves the N-terminus of the hepatitis C virus core protein

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## Abstract

Aminoacylase 3 (AA3) mediates deacetylation of N-acetyl aromatic amino acids and mercapturic acids. Deacetylation of mercapturic acids of exo- and endobiotics are likely involved in their toxicity. AA3 is predominantly expressed in kidney, and to a lesser extent in liver, brain, and blood. AA3 has been recently reported to interact with the hepatitis C virus core protein (HCVCP) in the yeast two-hybrid system. Here we demonstrate that AA3 directly binds to HCVCP ($K_d$~10 μM) that may by implicated in HCV pathogenesis. AA3 also revealed a weak endopeptidase activity towards the N-terminus of HCVCP.

### Keywords

aminoacylase 3; hepatitis C virus core protein; endopeptidase

## 1. Introduction

Hepatitis C virus (HCV) affects ~3% of the world population [1]. HCV infection is associated with liver steatosis, cirrhosis, hepatocellular carcinoma, cryoglobulinemia, insulin resistance and diabetes 2 mellitus. The HCV core protein (HCVCP) plays an important role in the HCV assembly. It is formed from a single polyprotein of about 3000 amino acids by host signal peptidase to yield p23, a 191 a.a. protein attached to the endoplasmatic reticulum (ER) membrane that is subsequently cleaved in the C-terminus generating a 177(179) aa.

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mature soluble protein p21 [2]. After the release from the ER membrane and trafficking to the cytoplasm, HCVCP associates with lipid droplets that is an important step in the HCV maturation [2,3]. The hydrophobic domain 2 (118–170 a.a.) is involved in the association of HCVCP with lipids, whereas the soluble basic N-terminal domain 1 (1–117 a.a.) interacts with the viral RNA that is responsible for the nucleocapsid formation [2]. A recent study [4] demonstrated that of basic residues located in two clusters (cluster 1, aa. 6–23; cluster 2, aa. 39–62) within the N-terminal 62 amino acids of HCVCP, only four basic amino acids residues of cluster 2 (Arg50, Lys51, Arg59, and Arg62) are essential for the production of infectious viral particles. Nevertheless mutation of these residues did not interfere with HCVCP subcellular localization, HCVCP–RNA interaction, or HCVCP oligomerization [4].

The N-terminal domain also interacts with a number of host proteins including transcription factors, receptors, and protein kinases [2,3,5–7]. The interaction of the N-terminal domain with the proteasome activator PA28$\gamma$ in the nucleus is involved in the degradation of HCVCP mediated by 20S proteasome [8,9].

Recently a new binding partner of HCVCP in the host cell was indentified using the yeast two-hybrid system, namely aminoacylase 3 (AA3) [10]. AA3 is expressed in the kidney, liver, brain, blood [11,12], and mediates the deacetylation of N-acetyl L-amino acids with aromatic side chains as well as the deacetylation of mercapturic acids (S-conjugates of N-acetyl cysteine) derived from the glutathione (GSH) dependent detoxification of exo- and endobiotics [13]. The deacetylation of N-acetylaromatic amino acids formed from protein catabolism is a salvage mechanism given that the majority of eukaryotic proteins is $N^{\alpha}$-terminally acetylated [14]. The AA3 mediated deacetylation of N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine, the excretable product of the GSH-dependent detoxification of trichloroethylene, in renal proximal tubules [15] is responsible for acute renal failure induced by trichloroethylene [12,13]. The AA3 mediated deacetylation of a mercapturic acid generated during the GSH-dependent detoxification of a toxic aldehyde 4-hydroxynonenal is neurotoxic [16]. Inhibition of AA3 completely protects neuronal cells from 4-hydroxynonenal toxicity [16].

AA3 is expressed in liver and blood; therefore the AA3–HCVCP interaction may potentially play a role in the HCV pathogenesis. Given the potential false-positive signals in the yeast two-hybrid system, we performed this study to determine whether AA3 and HCVCP proteins interact.

## 2. Materials and methods

### 2.1. Reagents and peptide synthesis

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) except Complete protease inhibitors cocktail that was purchased from Roche (Indianapolis, IN, USA). The following peptides were synthesized: STLPKPQRKT (HCVCP-short) and STLPKPQRKTKRNTIRRPQDVKFPGG (HCVCP-long) corresponding respectively to aa. 2–11 and aa. 2–27 in HCVCP, SSLPGSREPL corresponding to aa. 2–11 in mouse AA3, and STENVEGKSPN corresponding to aa. 2–12 in the human sodium bicarbonate cotransporter NBCe1-A (SCL4A4). The N-terminally acetylated (*Ac*) versions of all peptides were also synthesized. The N-terminal acetylation of these proteins (after the cleavage of a methionine) was either predicted [17] or was demonstrated experimentally for the HCVCP expressed in insect cells [18]. The purity of all peptides was 95.7–98.1% as was confirmed by HPLC and mass-spectrometry (MS).

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### 2.2. Cloning, expression and purification of mouse AA3

Mouse AA3, with high structural and sequencing homology (with human AA3 [11,12,16], was used in this study. Mouse AA3 was expressed in *E. coli* as an N-terminally Strep(II)-tagged protein using the pRSET vector (Invitrogen, Carlsbad, CA, USA). Sequences of all constructs were confirmed by bi-directional sequencing using an ABI 310 sequencer (Perkin Elmer, Foster City, CA, USA). *E. coli* was grown to the optical density of 0.6, and then 1 mM isopropyl β-D-thiogalactopyranoside was added. Three hours later, bacterial cells were precipitated by centrifugation at 6000 g for 20 min and resuspended in PBS. The procedure was repeated twice, and the cells were disrupted in a BugBuster™ HT Protein Extraction Reagent (Novagen, Madison, WI, USA) containing Complete protease cocktail (Roche). Mouse AA3 was purified using affinity chromatography on Streptactin Sepharose (GE HealthCare, Piscataway, NJ, USA) in the presence of Roche complete protease inhibitor cocktail. The enzyme was eluted from a Streptactin Sepharose column with 3 mM desthiobiotin in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and desalted on a PD MiniTrap G-25 column (GE Healthcare). Purified AA3 was >99% purity judged by SDS-PAGE.

### 2.3. Cloning, expression and purification of HCVCP

The HCVCP cDNA coding aa. 1–177 was recloned from the pcDNA 3.1 vector containing HCVCP genotype 1 provided by Warren Schmidt (University of Iowa) to the pRSET vector (Invitrogen, Carlsbad, CA, USA) as a C-terminally 6xHis tagged protein and expressed in *E. coli*. The expressed HCVCP was solubilized in 9 M urea in the presence of Roche protease inhibitors cocktail, and purified using metal affinity chromatography on a Ni IMAC FF column (GE HealthCare). Protein was eluted with 200 mM imidazole in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.03 % dodecyl β-D-maltoside, and desalted on PD MiniTrap G-25 column.

### 2.4. Aggregation of AA3 with HCVCP and electron microscopy study of aggregates

Equal volumes of 0.1 mM solutions of mouse AA3 and HCVCP in 0.1 M Tris-HCl, pH 7.5, were mixed. The solution was absorbed on electron microscopic grids immediately after mixing. Grids were washed with milliQ $H_2O$ and negatively stained with 1% uranyl acetate. Micrographs were recorded on a 4k×4k CCD camera at 30000–200000× magnification in a FEI Tecnai F20 electron microscope operated at 200 kV.

### 2.5. Determination of the binding constants of HCVCP and peptides to AA3

The affinity of HCVCP and peptides to mouse AA3 was measured using the surface plasmon resonance method in a Biacore T100 (GE HealthCare). AA3 was immobilized on a CM5 Biacore chip using matrix activation by N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1:1 mixture). 50 mM Na-phosphate buffer, pH 7.5, was used as running buffer. Mouse AA3 (10 μg/ml in running buffer) was captured on flow cell 2 at a density of 300 response units at a flow rate 5 μl/min. HCVCP and peptides were injected over flow cells 1 and 2 at a flow rate 30 μl/min. The complex was allowed to associate and dissociate for 150 s and 420 s, respectively. The surface was regenerated with 50 μl of 1 M NaCl (for peptides) or 50 mM NaOH (for HCVCP) in running buffer. Various concentrations of peptides (1 μM – 10 mM) and HCVCP (0.01 μM – 1 mM) were injected over both flow cells, and the response curve on the flow cell 1 was subtracted from the flow cell 2. The data were fitted by the Kinetics/Affinity analysis with the Biacore T100 evaluation software, version 1.1.

### 2.5. Analysis of the peptide cleavage catalyzed by AA3

The reaction assay contained 3 μM AA3, 1 mM peptide or 0.1 mM HCVCP in 50 mM Tris-HCl, pH 7.5, and 1 mM $CoCl_2$. Cobalt (II) was added to the assay because it has been shown

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to significantly activate AA3 [12]. After incubation for 18 h at 37°C, an aliquot was diluted in milliQ $H_2O$ to a final peptide concentration 50 μM and injected (100 μl/injection) onto a reverse phase HPLC column (polymeric resin, 150 × 1 mm, 5 Å, Phenomenex, Torrance, CA, USA) equilibrated in water/formic acid (100/0.1, v/v) and eluted (50 μl/min) with an increasing concentration of acetonitrile (time/% acetonitrile: 0/0, 60/100). The effluent from the column was passed in series through a fixed wavelength UV detector (215 nM) and then an Ionspray™ source interfaced with a triple quadrupole mass spectrometer (PE Sciex API III+) that was scanning from m/z 300–2000 (orifice 65 volts, 6 secs/scan). Accession of spectra and deconvolution of the multiply charged ion clusters into true molecular weight spectra was performed using the instrument supplied sofware (MacSpec™ version 3.3, PE Sciex). Cleavage sites in the peptides were determined using the EXPASY FindPept tool (http://www.expasy.org/tools/findpept.html).

### 2.6. AA3 deacetylating assay

The deacetylating activity of AA3 towards the $N^{\alpha}$-acetylated peptides was determined in an assay, which in a total volume 2 ml contained 1 mM peptide, 3 μM AA3 in 50 mM Tris-HCl, pH 7.5, with or without 0.1 mM $CoCl_2$. The reaction was started by adding AA3. Aliquots of 500 μl each were taken after 0, 1, 2 and 3 h incubation at 37°C, and analyzed using an Acetate detection kit (R-Biopharm, Darmstadt, Germany) as recommended by the manufacturer. The boiled for 2 min wt-AA3 was used in the control experiments.

## 3. Results

### 3.1. Peptide deacetylation by AA3

AA3 did not deacetylate any of the N-terminally acetylated peptides used in this study, namely *Ac*-HCVCP-short, *Ac*-HCVCP-long, *Ac*-AA3 and *Ac*-NBCe1-A. This is in agreement with the preferential deacetylation by AA3 of N-acetyl aromatic not aliphatic amino acids. In addition, no corresponding deacetylated peptides were detected in the assay using a LC-MS technique (data not shown).

### 3.2. Binding of HCVCP and peptides to AA3

Precipitation was observed immediately after mixing of AA3 with HCVCP in support of the direct interaction of these proteins. Individual proteins did not demonstrate any aggregation (Fig. 1, left). HCVCP particles of 2–3 nm are seen that probably represent different projection of HCVCP monomers and/or dimers. In agreement with a previous study [19], AA3 is mainly represented by dimers. A heterogeneous population of round particles of different size (20–60 nm) assembled in significantly larger (>1500 nm) irregular branched structures was seen under electron microscope in the suspension formed after mixing AA3 with HCPCP (Fig. 1, right).

To quantitate the interaction of HCVCP with AA3 we used the surface plasmon resonance method. The $K_d$ value for interaction of HCVCP with AA3 was 10.1±4.3 μM (Table 1). A moderately high affinity of HCVCP to AA3 suggests that they may physically interact in the organs/tissues where they are co-expressed, for example in the liver and blood.

To locate the HCVCP region responsible for this interaction and given that interactions of the core protein with different protein partners are mostly mediated by its extreme N-terminal part [2,4], we synthesized short (STLPKPQRKT) and long (STLPKPQRKTKRNTIRRPQDVKFPGG) HCVCP peptides corresponding to aa. 2–11 (HCVCP-short) and 2–27 (HCVCP-long).

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N-acetylated HCVCP-short peptide (*Ac*-HCVCP-short) had the $K_d$ value of 7.3 mM, whereas the non-acetylated short peptide bound to AA3 with a higher affinity ($K_d$=1.6 mM). Both *Ac*-HCVCP-long and HCVCP-long peptides demonstrated higher affinities to AA3 (170±13 and 142±11 respectively) than the corresponding short peptides but lesser affinities than the full-length HCVCP (Table 1). Again N-acetylation decreased the affinity of long peptide. The data suggested that the N-terminal acetylation impaired the binding of a peptide to AA3 likely via decreasing of the pI value of the acetylated peptide. Indeed the N-acetylation of HCVCP-short decreases its predicted pI value from 11.17 to 10.06, and HCPCP-long from 12.02 to 11.58. To further confirm this hypothesis, we synthesized two peptides with the pI values below 6, namely SSLPGSREPL corresponding to aa. 2–11 in mouse AA3 (calculated pI=5.72), and STENVEGKSPN corresponding to aa. 2–12 in the human electrogenic sodium bicarbonate cotransporter NBCe1-A (calculated pI=4.53). Both non-acetylated and N-acetylated peptides were used. Again as with HCVCP peptides, acetylated peptides of AA3 and NBCe1-A were used because of the predicted acetylation of the second residue (Ser2) after the cleavage of a methionine [17]. Both acetylated and non acetylated AA3 and NBCe1-A peptides demonstrated poor binding to the enzyme ($K_d$>10 mM). Figure 2 illustrates the dependence of the affinity of peptides to AA3 from their pI values. The $K_d$ value is significantly decreased at the peptide pI>10.

Although the $K_d$ values for peptide–AA3 interactions were quite similar to the $K_m$ values of AA3 substrates [12,13], and the binding of the peptides to the active site of AA3 could not be excluded, the presence of several exposed negatively charged areas in the mouse AA3 atomic structure [20] suggests that multiple sites on the surface of AA3 may be involved in the interaction with HCVCP.

### 3.3. Peptide cleavage by AA3

The binding of AA3 to HCVCP peptides suggested that AA3 may potentially deacetylate them although the substrate specificity of AA3, which deacetylates N-acetyl aromatic amino acids and mercapturic acids (N-acetyl-S-conjugates of L-cysteine) with bulky side chains [13], did not support this suggestion. In agreement with the substrate specificity, AA3 did not generate acetate during even 24 h incubation with N-acetylated HCVCP and other peptides. Instead, a peptide hydrolysis was detected using LC/MS (Fig. 3A–D). Three peptides (STLPKP, STLPKPQ and STLPKPQR) were detected after incubation of both N-acetylated and non-acetylated short HCVCP peptide (STLPKPQRKT). No C-terminal peptides were detected. The results suggested that AA3 has endopeptidase activity towards the HCVCP peptides. This is in agreement with the structural similarity of the active site of AA3 and carboxypeptidase A [20]. Surprisingly, both *Ac*-HCVCP-long and HCVCP-long were not hydrolyzed by AA3. Given the higher affinity of HCVCP-long than HCVCP-short to AA3, the larger size of HCVCP-long may prevent its productive orientation towards the enzyme peptidase site. Both the N-acetylated and non-acetylated AA3 peptide (SSLPGSREPL) were hydrolyzed between Gly5 and Ser6 and both moieties (SSLPG and SREPL) were present (Fig. 3E–H). Both acetylated and non-acetylated NBCe1-A peptides were not hydrolyzed (data not shown). The endopeptidase activity of AA3 was $Co^{2+}$-dependent; it was not detected without this metal ion. The endopeptidase activity of AA3 was likely attributed to the enzyme active center since the inactive H21A mutant of AA3 did not hydrolyze the peptides (data not shown). The endopeptidase activity of AA3 was not due to protease contaminants since standard protease substrates insulin B chain and casein were not hydrolyzed (data not shown). The $Co^{2+}$-dependent endopeptidase activity of AA3 was inhibited by ~50% in the presence of 1 mg/ml Pefabloc SC suggesting that a serine residue(s) may be involved in mediating this activity. It should be mentioned that a serine residue is not present in the hypothetical active site of mouse AA3 [20] although 20 serine residues are present in AA3 monomer [11,12].

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## Discussion

Our study for the first time demonstrates that HCVCP directly interacts with AA3. This interaction is mediated by the highly basic extreme N-terminus of HCVCP. The interaction between AA3 and HCVCP may play an important role in HCV pathogenesis. Binding of AA3 to the HCV capsid may form a specific shield protecting the virus from the recognition by the host immune system. From this standpoint it is important to determine whether AA3 is present on the surface of the HCV particles *in vivo*.

Our study provides the first evidence for the presence of endopeptidase activity in a mammalian aminoacylase. Dipeptidase and carboxypeptidase activity of several microbial aminoacylases have been reported [21–24]. Cleavage of the neighbor amino acids in the C-terminus of HCVCP-short suggests that AA3 also possesses carboxypeptidase activity. This is in a good agreement with the similarity of the crystal structure and catalytic mechanism of AA3 and carboxypeptidase A [20]. AA3 does not demonstrate preferences for amino acids in the position P1 and P1' of the substrate since the enzyme cleaves the peptide bonds between both charged and uncharged amino acids. The cleavage occurs only in the C-terminal and central part of peptides not in their N-terminus. The net charge of the N-terminal part of peptides seems to be important for their cleavage by AA3 with the preference to a positive charge. The N-terminus of the best hydrolyzed peptide, HCVCP, has a positive charge, the N-terminus of a significantly less hydrolyzed AA3 peptide is near neutral, while the non-hydrolyzed NBCe1-A peptide has a negative charge in the N-terminal half. The absence of the hydrolytic activity of AA3 towards the higher affinity HCVCP-long peptide and whole HCVCP argues for the existence of additional binding site(s) distinct from the enzyme active site. In agreement with this hypothesis, if the hydrolysis occurs, the dissociation of products should be significantly decelerated. According to the N–end rule, the N-terminal amino acids with a bulky or charged side chain are considered to be destabilizing and target proteins to the site of intracellular ubiquitin–dependent proteolysis [25]. The ubiquitin–mediated degradation of the HCVCP was demonstrated previously [9,26,27]. Cleavage of the N-terminus of HCVCP mediated by AA3 may expose destabilizing amino acids and affect the turnover rate of HCVCP in the cell.

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## Abbreviations

| | |
|---|---|
| **AA3** | aminoacylase 3 |
| **ER** | endoplasmatic reticulum |
| **HCV** | hepatitis C virus |
| **HCVCP** | hepatitis C virus core protein |
| **MS** | mass-spectrometry |
| **NBCe1-A** | the kidney specific form of the electrogenic sodium bicarbonate cotransporter 1 |

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**Highlights**

- AA3 hydrolyzes the N-terminal decapeptide of hepatitis C core protein
- AA3 binds to hepatitis C core protein
- AA3 induces aggregation of hepatitis C core protein

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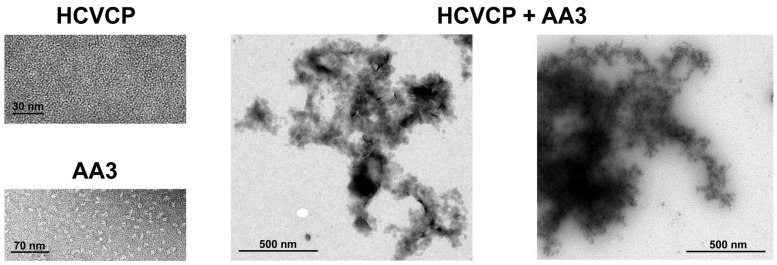


**Fig. 1.**

Electron micrographs of negatively stained mouse AA3–HCVCP aggregates. Electron micrographs of AA3 and HCVCP solutions that were used to obtain AA3–HCVCP aggregates are shown for comparison.

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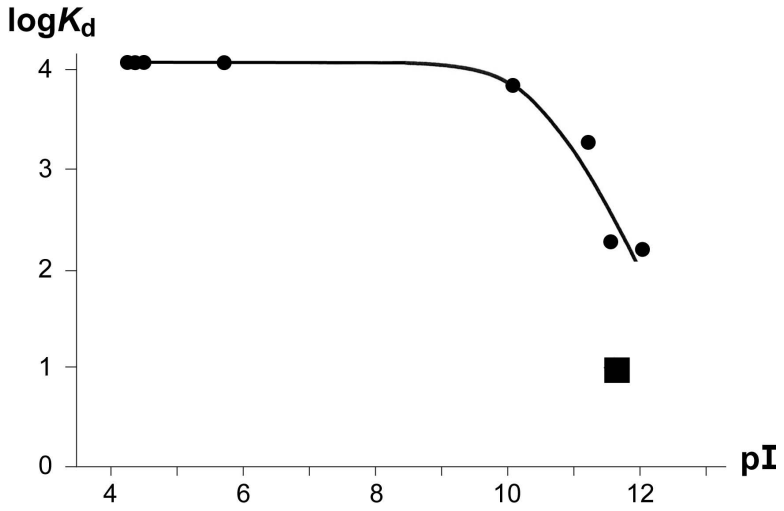


**Fig. 2.**

Effect of the peptide isoelectric point (pI) on the strength of binding ($K_d$) to mouse AA3. Black circles: peptides. Black square: HCVCP.

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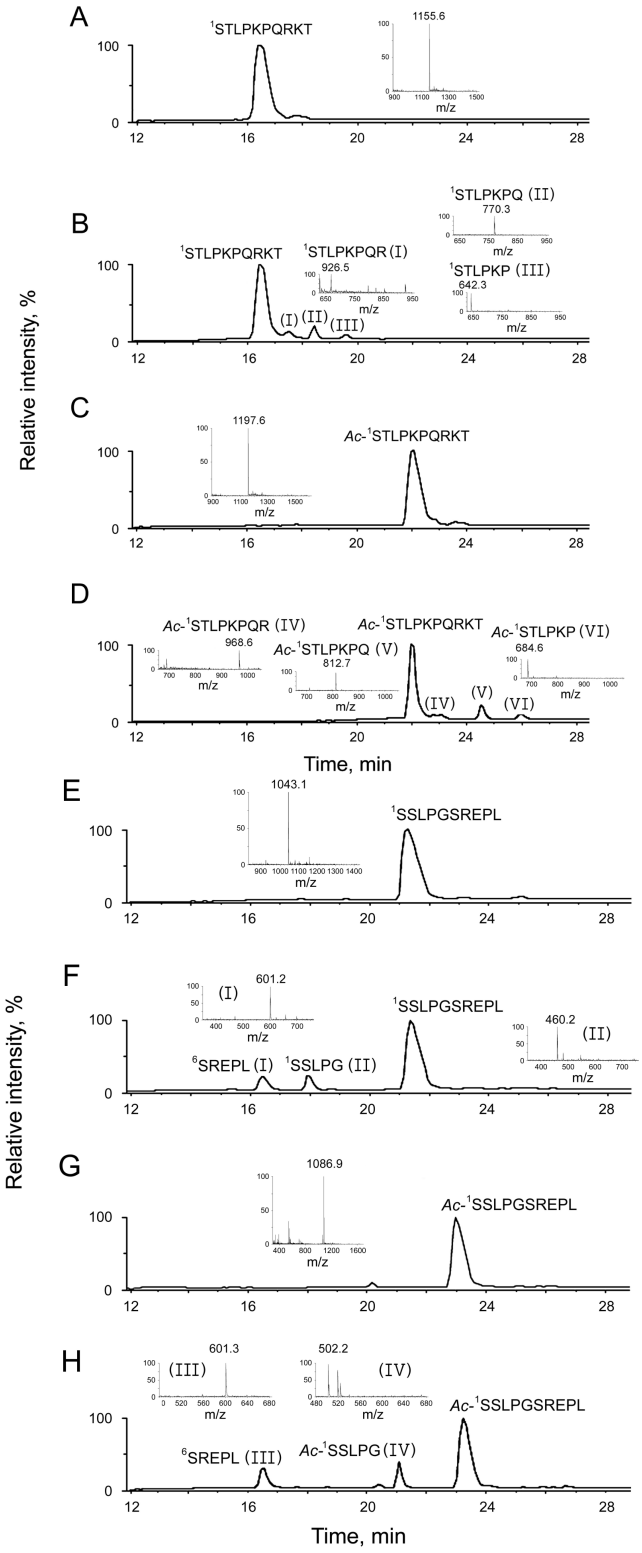


**Fig. 3.**

LC-MS analysis of HCVCP-short (A,B), Ac-HCVCP-short (C,D), AA3-short (E,F) and *Ac*-AA3-short (G,H) incubated with the intact (B,D,F,H) and boiled (A,C,E,G) mouse AA3.

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## Table 1

Affinity of the HCV core protein and its N-terminal peptides to mouse AA3. (*Ac*) shows the N-terminal acetylation. Binding was measured using the surface plasmon resonance method in a Biacore T100 (GE HealthCare).

| Peptide | $K_d$, μM | Calculated pI |
|---|---|---|
| HCVCP (1–177 a.a.) | 10.1±4.3 | 11.64 |
| Short *Ac*-HCVCP peptide (2–11 a.a.) | 7300±370 | 10.06 |
| Short HCVCP (2–11 a.a.) | 1580±200 | 11.17 |
| Long *Ac*-HCVCP (2–27 a.a.) | 170±13 | 11.58 |
| Long HCVCP (2–27 a.a.) | 142±11 | 12.02 |
| *Ac*-AA3 peptide (2–11 a.a.) | >10000 | 4.37 |
| AA3 peptide (2–11 a.a.) | >10000 | 5.72 |
| *Ac*-NBCel-A peptide (2–12 a.a.) | >10000 | 4.25 |
| NBCel-A peptide (2–12 a.a.) | >10000 | 4.53 |