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ORIGINAL ARTICLE

On the localization of the cleavage site in human alpha-2-antiplasmin, involved in the generation of the non-plasminogen binding form

Shiraazkhan Abdul[1] | Dick H. W. Dekkers[2] | Robert A. S. Ariëns[3] | Frank W. G. Leebeek[1] | Dingeman C. Rijken[1] | Shirley Uitte de Willige[1]

[1]Department of Hematology, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands

[2]Center for Proteomics, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands

[3]Thrombosis and Tissue Repair Group, Leeds Institute of Cardiovascular and Metabolic Medicine, School of Medicine, University of Leeds, Leeds, UK

Correspondence
Dingeman C. Rijken, Department of Hematology, Erasmus MC, University Medical Center Rotterdam, Wytemaweg 80, 3015 CN Rotterdam, the Netherlands.
Email: d.rijken@erasmusmc.nl

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Abstract

Background: Alpha-2-antiplasmin (α2AP) is the main natural inhibitor of plasmin. The C-terminus of α2AP is crucial for the initial interaction with plasmin(ogen) and the rapid inhibitory mechanism. Approximately 35% of circulating α2AP has lost its C-terminus (non-plasminogen binding α2AP/NPB-α2AP) and thereby its rapid inhibitory capacity. The C-terminal cleavage site of α2AP is still unknown. A commercially available monoclonal antibody against α2AP (TC 3AP) detects intact but not NPB-α2AP, suggesting that the cleavage site is located N-terminally from the epitope of TC 3AP.

Objectives: To determine the epitope of TC 3AP and then to localize the C-terminal cleavage site of α2AP.

Methods: For epitope mapping of TC 3AP, commercially available plasma purified α2AP was enzymatically digested with Asp-N, Glu-C, or Lys-N. The resulting peptides were immunoprecipitated using TC 3AP-loaded Dynabeads® Protein G. Bound peptides were eluted and analyzed by liquid chromatography-tandem mass spectometry (LC-MS/MS). To localize the C-terminal cleavage site precisely, α2AP (intact and NPB) was purified from plasma and analyzed by LC-MS/MS after enzymatic digestion with Arg-C.

Results: We localized the epitope of TC 3AP between amino acid residues Asp428 and Gly439. LC-MS/MS data from plasma purified α2AP showed that NPB-α2AP results from cleavage at Gln421-Asp422 as preferred site, but also after Leu417, Glu419, Gln420, or Asp422.

Conclusions: The C-terminal cleavage site of human α2AP is located N-terminally from the TC 3AP epitope. Because C-terminal cleavage of α2AP can occur after multiple residues, different proteases may be responsible for the generation of NPB-α2AP.

KEYWORDS

alpha-2-antiplasmin, epitope mapping, mass spectrometry, proteolysis, western blot

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1 | INTRODUCTION

Alpha-2-antiplasmin (α2AP) is the main natural inhibitor of the fibrinolytic enzyme plasmin.[1-3]

Native α2AP is produced by the liver and circulates in blood as an approximately 67-kDa single-chain glycoprotein of 464 amino acid residues, containing 11% to 14% carbohydrate.[2,4,5] α2AP possesses unique N- and C-terminal ends, as we extensively reviewed.[6] The N-terminus is involved in α2AP incorporation into a blood clot by activated factor XIII-mediated crosslinking.[7,8] The C-terminus is crucial for the initial interaction of α2AP with plasmin(ogen).[9] α2AP contains six lysine residues at its C-terminus, which are involved in the interaction with lysine binding sites in plasminogen.[9,10] Based on the interaction with these lysine binding sites, two molecular species of α2AP have been described in plasma.[11] One molecular form is able to bind to plasminogen (plasminogen binding α2AP, PB-α2AP), comprises approximately 65% of circulating α2AP, and is a fast-acting inhibitor of plasmin by rapidly forming plasmin-antiplasmin complexes. The other molecular form, which comprises approximately 35% of circulating α2AP, does not bind to plasminogen (non-plasminogen binding α2AP, NPB-α2AP) and inhibits plasmin at a much slower rate.[11,12] Kluft and Los demonstrated the presence of the two variants in plasma and in serum by modified crossed immunoelectrophoresis.[13] They also showed that only PB-α2AP is produced in the liver and that NPB-α2AP is the result of posttranslational modification in the circulation.[14] Furthermore, it was reported that the NPB-α2AP fraction in plasma may contain some inactive or inactivated inhibitor.[11,15]

In addition to the main form of NPB-α2AP in vivo, it was shown that purified PB-α2AP can spontaneously convert into an NPB-α2AP form in vitro.[16] Sasaki et al[17] treated both purified PB-α2AP and in vitro formed NPB-α2AP after acetamidination with trypsin and analyzed the peptides by HPLC. A peptide representing the 26 C-terminal amino acids of the molecule was found in PB-α2AP, which was not present in NPB-α2AP formed in vitro. This indicated the participation of this C-terminal part in plasminogen binding. Some subsequent papers have mistakenly considered this trypsin cleavage site as the position where PB-α2AP is truncated into NPB-α2AP.[18,19] The study by Sasaki et al only showed that NPB-α2AP is formed in vitro after a cleavage of a peptide smaller than 26 amino acid residues. To date, it is unknown where and how the conversion happens in the circulation. An actual in vivo cleavage site and a protease responsible have not yet been identified.

Leebeek et al[20] have suggested that the in vivo and in vitro conversions of PB- α2AP to NPB-α2AP in plasma may have different mechanisms and possibly involve different cleavage sites. Using a commercially available monoclonal antibody against the C-terminus of α2AP (TC 3AP[21]) in a crossed immunoelectrophoresis technique, they showed that NPB-α2AP formed in vivo lost its capacity to bind to both plasminogen and the antibody, whereas NPB-α2AP formed in vitro lost the plasminogen binding capacity, but could still bind to the antibody. This indicated that in vivo and in vitro generated NPB-α2AP are not the same molecules but may differ in their C termini or may be structurally different. We reckoned that the epitope recognized by TC 3AP in the α2AP protein can be used to obtain a crude localization of the in vivo C-terminal cleavage site of α2AP; therefore, this study aimed to determine the epitope of TC 3AP and subsequently localize the C-terminal cleavage site of α2AP by mass spectrometry.

Essentials

- Due to proteolytic cleavage, part of circulating a2-antiplasmin (α2AP) loses the C-terminus.
- C-terminally cleaved α2AP is unable to inhibit plasmin rapidly.
- We found that C-terminal cleavage of α2AP occurs between residues Leu417 and Asp422.
- Our study gives more insight into the regulation of C-terminal heterogeneity of α2AP.

2 | METHODS

2.1 | Materials

Pooled normal plasma was prepared from citrated apheresis plasma (Sanquin blood bank, Rotterdam, The Netherlands) of five healthy donors. Commercially available plasma-purified α2AP (Calbiochem) was purchased from Merck Millipore. The mouse monoclonal antibody against the C-terminus of α2AP (TC 3AP) was purchased from Technoclone. The custom made polyclonal rabbit Asn-α2AP antibody (anti-Asn-α2AP, affinity purified IgG) was raised against a peptide corresponding to amino acids Gln14 to Pro30 of the α2AP protein (Charles River Laboratories, Squarix GmbH) and has been used previously.[22] The polyclonal goat total α2AP antibody (affinity purified IgG) (anti-total α2AP) directed to all forms of α2AP and the horseradish peroxidase-conjugated anti-total α2AP antibody were purchased from Affinity Biologicals. Cyanogen bromide-activated-Sepharose® 4B was purchased from GE Healthcare. Sigma*FAST*™ Protease Inhibitor Cocktail, ammonium bicarbonate, 2-chloroacetamide, 1,4-dithiothreitol (DTT), and 3,3′,5,5′-tetramethylbenzidine were purchased from Sigma-Aldrich. Magnetic Dynabeads® Protein G, GelCode® Blue Stain Reagent, and endoproteinase Glu-C were purchased from Thermo Fisher Scientific. Endoproteinase Arg-C was purchased from Protea. Endoproteinase Asp-N was obtained from Roche. Endoproteinase Lys-N was purchased from U-Protein Express BV. XT Sample Buffer, XT reducing agent, and precast XT Criterion 10% polyacrylamide gels were purchased from BioRad.

2.2 | Epitope mapping of antibody TC 3AP

Epitope mapping of TC 3AP was carried out according to an adapted epitope extraction method as described previously.[23,24] In detail, commercially available plasma-purified α2AP, lyophilized in buffer (200 mmol/L NaCl, 20 mmol/L Bis-Tris, pH 6.4), was resuspended in

distilled water followed by addition of Tris-HCl (pH 8.5) (50 mmol/L final concentration). Reduction and alkylation of α2AP was carried out by adding DTT (5 mmol/L final concentration), followed by incubation for 30 minutes at 50°C under constant motion. Next, 2-chloroacetamide was added (10 mmol/L final concentration) followed by incubation for 30 minutes at room temperature (RT) in the dark. The reaction was quenched by the addition of extra DTT (2.5 mmol/L final concentration), followed by digestion of α2AP with Asp-N, Glu-C, or Lys-N. A 1:20 final ratio (w/w) of Asp-N or Glu-C with α2AP, and a 1:1 final ratio of Lys-N with α2AP was used (buffers: 50 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, pH 8.5 for Asp-N; 50 mmol/L Tris-HCl, pH 8.5 for Glu-C; 100 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, pH 10 for Lys-N). The mixtures were incubated overnight at 30°C. The digestion reactions were terminated by boiling the mixtures for 10 minutes at 95°C.

After enzymatic digestion, α2AP peptides containing the epitope were purified from the mixtures by immunoprecipitation with TC 3AP coupled magnetic Dynabeads® Protein G. The coupling procedure was carried out by diluting 20 µg TC 3AP with phosphate-buffered saline with Tween (PBST; 137 mmol/L NaCl, 2.7 mmol/L KCl, 6.5 mmol/L Na_2HPO_4, 1 mmol/L KH_2PO_4, 0.02% Tween-20, pH 7.4), followed by incubation of the diluted antibody solution with 3 mg magnetic Dynabeads® Protein G for 1 hour at 4°C under constant motion. The beads were separated from the solution using a magnet, followed by removal of the supernatant. The beads were washed with PBST, mixed with the digested α2AP, and incubated overnight at 4°C under constant motion. After three stringent wash steps with PBST and five wash steps with phosphate buffered saline (PBS), bound α2AP peptides were eluted from the beads using 100 mmol/L glycine buffer (pH 2.2) during incubation for 1 hour at RT under constant motion. Eluted peptides were collected, desalted using ZipTip C18 according to the manufacturer's instructions, dried, and analyzed by liquid chromatography-tandem mass spectrometry.

2.3 | Purification of α2AP

For the localization of the C-terminal cleavage site, PB- and NPB-α2AP were purified from normal pooled plasma by affinity chromatography. Briefly, 1 mg anti-total α2AP was coupled to 1 mL cyanogen bromide-activated-Sepharose® 4B. Normal pooled plasma (6 mL) was diluted with 6 mL wash buffer (0.1 mol/L Tris-HCl, 0.1% Tween-20, 1 mol/L NaCl, pH 8.1). To prevent nonspecific cleavages in α2AP, Protease Inhibitor solution was added to the diluted plasma sample, wash buffer and elution buffer (100 mmol/L glycine, 0.01% Tween-20, 0.1 mol/L NaCl, pH 2.2). The diluted plasma sample was incubated overnight with the anti α2AP-IgG-Sepharose 4B gel at 4°C with gentle stirring. After incubation, the gel was washed four times with wash buffer and placed in a column. α2AP was eluted with 4 mL elution buffer. The eluted fractions were neutralized with 1 mol/L Tris-HCl, pH 9.0.

The α2AP concentration of the eluted fractions was determined by ELISA. 96-Well microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with 2 µg/mL (110 µL $well^{-1}$ in 0.05 mol/L carbonate buffer, pH 9.6) anti-total α2AP antibody overnight at 4°C. Nonspecific sites were blocked with 200 µL 1% BSA in PBS containing 0.002% Tween-20 for 1 hour at RT and plates were washed once with PBS/0.002% Tween-20. Subsequently, eluted fractions were diluted 40 times and serial dilutions were made of normal pooled plasma (1:200-1:12 800) for the standard curve. Plates were incubated for 2 hours at RT followed by four rounds of washing with PBS/0.002% Tween-20. Next, a horseradish peroxidase-conjugated anti-total α2AP antibody (1:2000) was added and incubated for 1 hour at RT. After four wash steps with PBS/0.002% Tween-20, enzyme activity was determined using 3,3′,5,5′-tetramethylbenzidine as substrate and the reaction was stopped after 10 minutes by adding 100 µL/well of 1 mol/L H_2SO_4. The absorbance was read at 450 nm on a Victor^3 1420 Multilabel Plate Counter (Perkin Elmer).

The eluted fractions with the highest α2AP concentrations were pooled and dialyzed against distilled water. Finally, the dialyzed pooled sample was dried by a SpeedVac procedure and resuspended in 60 µL distilled water.

2.4 | SDS-PAGE and Western blotting

To separate PB-α2AP and NPB-α2AP, 5 µg (for SDS-PAGE) and 300 ng (for Western blot analyses) affinity-purified α2AP in

TABLE 1 Epitope mapping of antibody TC 3AP

Sequence	Amino acids	Frequency	Mass	m/z	Dev	Score
K.DFLQSLKGFPRG.D	428-439	11	1363.7248	455.5815	−1.57	39
K.DFLQSLKGFPRG.D	-	-	-	682.8696	−0.18	51
Q.DSPGNKDFLQSLKGFPRG.D	422-439	12	1961.9959	491.5056	−1.44	41
Q.DSPGNKDFLQSLKGFPRG.D	-	-	-	655.0053	−1.02	57
Q.DSPGNKDFLQSLKGFPRG.D	-	-	-	982.0041	−1.11	61

Notes: Dev, the difference between the observed and calculated mass (in ppm); frequency, the number of times a peptide is observed in the mass spectrometry analysis; mass: the theoretical monoisotopic mass of the neutral peptide (in Daltons); m/z, the observed mass/charge; score, the ions score given to the peptide.

Peptides identified by Mascot analysis in the immobilized TC 3AP-bound α2AP fraction produced by enzymatic digestion of α2AP with Asp-N. Best scoring spectra are reported.

Tris-buffered saline were boiled for 5 to 10 minutes at 95°C in the presence of XT Sample Buffer and XT reducing agent and resolved on a precast XT Criterion 10% polyacrylamide gel. After electrophoresis, the proteins were stained by Coomassie or transferred to a nitrocellulose membrane using a PowerPac™ HC power supply (BioRad, Richmond, CA) with transfer buffer (25 mmol/L Tris, 192 mmol/L glycine [pH 8.3], and 20% methanol) at 100V for 1 hour. After protein transfer, nonspecific sites on the nitrocellulose membrane were blocked with 5% milk in PBS, pH 7.4, followed by three wash steps with PBS/0.1% Tween-20. Postblocking, the blot was incubated with a mixture of TC 3AP (1 mg/mL, 1:5000) and anti-Asn-α2AP (1 mg/mL, 1:5000) in PBS/0.1% Tween-20 overnight at 4°C under constant motion. After three wash steps, IRDye® 680CW donkey anti-mouse secondary antibody and IRDye® 800CW donkey anti-rabbit secondary antibody were used for detection of TC 3AP and the anti-Asn-α2AP antibody, respectively. After incubation for 1 hour at RT, three wash steps were performed and the blot was scanned in the 680- or 800-nm channel of an Odyssey® Imaging System (Lincoln, NE).

2.5 | Mass spectrometry for epitope mapping and C-terminal cleavage site analysis

For epitope mapping, the dried peptides containing the TC 3AP epitope were dissolved in 3% acetonitrile (ACN) with 0.5% formic acid (FA) and briefly sonicated. A fraction was injected onto a Waters nanoAcquity LC equipped with a Waters 20 mm x 180 µm nanoACQUITY UPLC Symmetry C18 Trap Column with 5-µm particles and a homemade 40 cm x 75 µm fused silica analytical column with Waters 3.5-µm XBridge BEH C18 particles. Peptides were separated using a 60-minute gradient from 99%A/1%B to 65%A/35%B (A = 0.1% FA, B = 0.1% FA in ACN) at 0.3 µL/min and 50°C and analyzed on a Thermo Orbitrap Fusion mass spectrometer in positive mode using a nanoESI source and a top speed method with 3 seconds' cycle time and HCD/ETD fragmentation, full scan detection at 120K resolution in the orbitrap, and fragment ion detection in the ion trap.

Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.3; MatrixScience). The Mascot algorithm was used for searching against a UniProt database (release 2015_03.fasta or newer, taxonomy: Homo sapiens). The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da for fragmentation spectra. A maximum number of two missed cleavages were allowed. Carbamidomethylated cysteine was set as fixed modification and oxidized methionine and deamidated asparagine and glutamine were set as variable modifications.

Data were additionally analyzed with Proteome Discoverer (version 1.4; Thermo Scientific) and MaxQuant (version 1.5.2.8) using similar settings[25] and appropriate digestion modes. For MaxQuant a false discovery rate of 0.01 for proteins and peptides and a minimum peptide length of seven amino acids were required. The tandem mass spectometry spectra were searched against the UniProt database concatenated with the reversed versions of all sequences.

For C-terminal cleavage site analysis, a pilot experiment was performed in which gel bands corresponding to PB-α2AP and NPB-α2AP were cut from the gel, divided into equal parts, and destained, reduced/alkylated, and dehydrated. The protein bands were subjected to overnight in-gel digestion at RT using 0.25 µg Arg-C in 50 mmol/L ammonium bicarbonate, 1 mmol/L $CaCl_2$ 1 mmol/L DTT pH 8, 0.05 µg Asp-N in 50 mmol/L Tris-HCl 1mM $CaCl_2$ pH 8, 0.1 µg Lys-C in 25 mmol/L Tris-HCl pH 8.5, or 0.2 µg trypsin in 50 mmol/L ammonium bicarbonate 1 mmol/L $CaCl_2$ pH 8.0. Peptides were extracted from the gel with 30% ACN 0.5% FA and mixing for 30 minutes in a shaker followed by sonication in a water bath for 2 minutes. The extracts were dried in a SpeedVac and the residues were redissolved in 3% ACN 0.5% FA and desalted with a ZipTip C18 according to the manufacturer's instructions. The final eluates were dried in a SpeedVac and redissolved in 3% ACN 0.5% FA and sonicated briefly. Aliquots were injected into the mass spectrometer as described previously.

After the pilot experiment, the C-terminal cleavage site analysis was repeated under optimized conditions in which bands on gel were quantified using a BioRad GS-900 Calibrated Densitometer and the NPB-α2AP band, calculated at 1 ug protein, was cut from the gel and pretreated as described previously. Protein was in-gel digested at 30°C overnight with 0.5 µg Arg-C in 50 mmol/L ammonium bicarbonate, 5 mmol/L $CaCl_2$, and 5 mmol/L DTT. Peptides were extracted, desalted, dried, and redissolved as described. An aliquot was injected onto a Thermo Scientific Easy-nLC 1200 equipped with a homemade 40 cm x 75 µm fused silica analytical column with Waters 3.5 µm XBridge BEH C18 particles without trapping column. Peptides were separated using a 60-minute gradient from 99%A/1%B to 50%A/50%B (A = 0.1% FA, B = 0.1% FA in 80% ACN) at 0.3 µL/min and 50°C and analyzed on a Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer in positive mode using a nanoESI source and a top speed method with 3 seconds' cycle time and HCD/

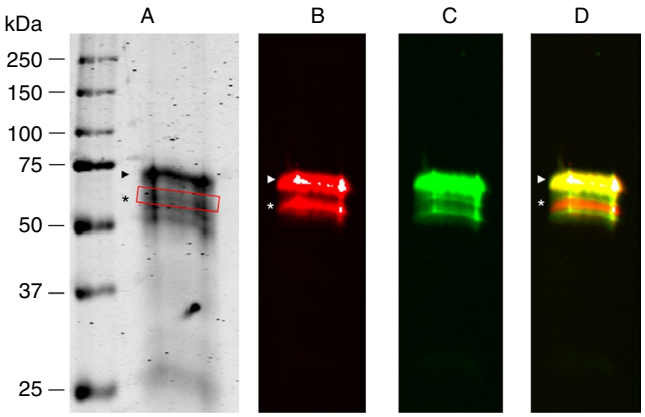


FIGURE 1 In-house purified α2AP visualized by SDS-PAGE and Western blot. (A) SDS-gel of purified α2AP. (B) Immunoblot obtained with anti-Asn-α2AP. (C) Immunoblot obtained with TC 3AP. (D) Merged anti-Asn-α2AP and TC 3AP blots. The relative migration distances of molecular weight marker proteins are indicated on the left. PB-α2AP is indicated by arrowhead. NPB-α2AP is indicated by an asterisk, as well as by a red box in (A)

EThcD fragmentation, full scan detection at 60K resolution in the orbitrap, and fragment ion detection in the ion trap. Expected NPB-α2AP C-terminal peptide masses, based on results from the pilot experiment, were calculated assuming charge states 2+ and 3+, up to 4 deamidations and up to 1 missed cleavage using sequences ELKEQQ, ELKEQQD, NPNSAPRELKEQQ, and NPNSAPRELKEQQD. These in total 26 m/z masses were favored for MS2 fragmentation by HCD and EThcD over other masses using an AGC target of 1E4 and increased injection times of 200 ms versus 50 ms for other masses (Table S1).

Data were analyzed with Mascot and MaxQuant (version 1.5.4.1) using similar settings as described and searched against the UniProt human database supplemented with truncated versions of α2AP (UniProt identifier P08697-1) starting from amino acid 300 up to 491 and concatenated with the reversed versions of all sequences. Numbering of α2AP amino acids was according to methionine in position 1.

3 | RESULTS

3.1 | Epitope mapping of antibody TC 3AP

To obtain a crude localization of the α2AP C-terminal cleavage site, we first determined the epitope of TC 3AP. Because this antibody does not react with NPB-α2AP, we hypothesized that the in vivo cleavage site is located N-terminally from the TC 3AP epitope. The MS data of the Asp-N digest identified two peptides that were bound to immobilized TC 3AP and that should contain the epitope of TC 3AP: Asp428-Gly439 (ions score: 39 and 51) and Asp422-Gly439 (ions score: 41, 57 and 61) (Table 1). Multiple ions scores were found for the same peptide, depending on their charge (2+, 3+, or 4+). The MS data of the Glu-C and Lys-N digests did not display any peptides, probably because of the presence of multiple protease specific cleavage sites in this region, resulting in small peptides that do not bind to immobilized TC 3AP or are undetectable by MS. Thus, we localized the epitope of TC 3AP to the region between Asp428 and Gly439.

3.2 | C-terminal cleavage site analysis

SDS-PAGE of the affinity purified α2AP showed protein bands with molecular weights between 50 and 67 kDa (Figure 1A). Western blot analyses showed reactivity of the anti-Asn-α2AP antibody to apparently two bands with molecular weights of approximately 60 kDa and 67 kDa (Figure 1B). Multiple protein bands within the 60- to 67-kDa range showed reactivity with TC 3AP (Figure 1C), indicating the presence of the C-terminus. However, there was one protein band at approximately 63 kDa that did not react with TC 3AP, suggesting the absence of the C-terminus, thus representing NPB-α2AP (red band in Figure 1D). The two minor bands below the main band of PB-α2AP in Figure 1C could possibly explained by deglycosylation, truncation at the N-terminus, or by the presence of isoform 2 originating from alternative splicing (www.uniprot.org).

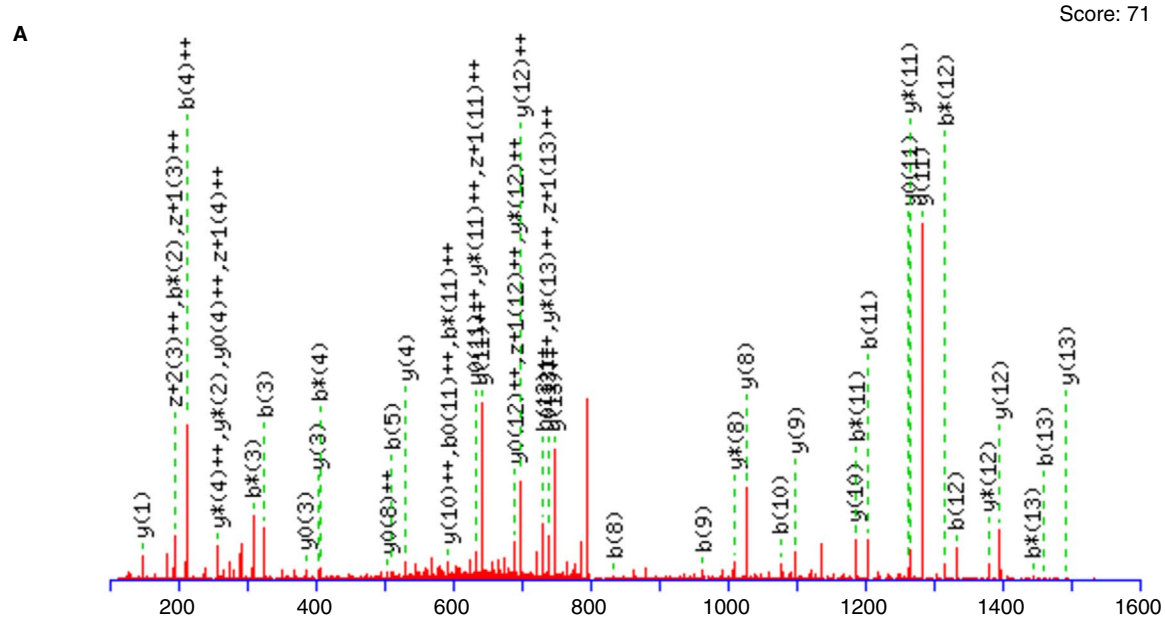
TABLE 2 NPB-α2AP peptides identified by Mascot and MaxQuant analyses after Arg-C digest (Figures S1-S6)

Sequence	Mascot analysis						MaxQuant analysis				
	Amino acids	Frequency	Mass	m/z	Dev	Score	Frequency	Mass	m/z	Dev	Score
R.NPNPSAPREL.K	408-417	1	1093.5518	547.7832	0.18	35	n.d.	n.d.	n.d.	n.d.	n.d.
R.NPNPSAPRELKE.Q	408-419	2	1350.6891	451.2374	0.89	34	n.d	n.d	n.d	n.d	n.d
R.NPNPSAPRELKEQ.Q	408-420	5	1478.7477	740.3826	2.03	58	1	1478.7478	740.3826	1.44	185
R.NPNPSAPRELKEQ.Q		-	-	-	-	-	1	-	494.2582	-2.63	101
R.NPNPSAPRELKEQQ.D	408-421	53	1606.8063	804.4133	3.62	71	25	1606.8063	536.60947	-2.14	193
R.NPNPSAPRELKEQQ.D		49	-	536.6099	0.91	55	-	-	-	-	-
R.NPNPSAPRELKEQQ.D[a]	408-421	24	1607.7903	804.9037	1.57	68	10	1607.7903	804.9047	2.21	234
R.NPNPSAPRELKEQQ.D[a]		19	-	536.9383	1.71	49	15	-	536.94424	1.96	187
R.NPNPSAPRELKEQQD.S	408-422	1	1721.8332	861.9233	-0.71	33	1	1721.8333	861.9233	0.19	163
R.NPNPSAPRELKEQQD.S		8	-	574.9523	1.01	61	6	-	574.9523	-1.27	213

Notes: Dev, the difference between the observed and calculated mass (in ppm); frequency, the number of times a peptide is observed in the mass spectrometry analysis; mass: the theoretical monoisotopic mass of the neutral peptide (in Daltons); m/z, the observed mass/charge; n.d., not determined; score, the ions score given to the peptide.

Best scoring spectra are reported.

[a]Deamidation.



#	b	b^{++}	b*	b*$^{++}$	b^{0}	b^{0++}	c	c^{++}	Seq.	w	y	y^{++}	y*	y*$^{++}$	y^{0}	y^{0++}	z+1	z+1^{++}	z+2	z+2^{++}	#
1	115.0502	58.0287	98.0237	49.5155			132.0768	66.5420	N												14
2	*212.1030*	106.5551	*195.0764*	98.0418			229.1295	115.0684	P	1450.7285	*1493.7707*	*747.3890*	1476.7441	*738.8757*	1475.7601	*738.3837*	1477.7520	*739.3796*	1478.7598	739.8835	13
3	*326.1459*	163.5766	*309.1193*	155.0633			343.1724	172.0899	N	1336.6856	*1396.7179*	*698.8626*	*1379.6914*	*690.3493*	1378.7074	*689.8573*	1380.6992	*690.8532*	1381.7070	691.3571	12
4	423.1987	*212.1030*	*406.1721*	203.5897			440.2252	220.6162	P	1239.6328	*1282.6750*	*641.8411*	*1265.6484*	*633.3279*	*1264.6644*	*632.8359*	1266.6563	*633.8312*	1267.6641	634.3357	11
5	*510.2307*	255.6190	493.2041	247.1057	492.2201	246.6137	527.2572	264.1323	S	1152.6008	*1185.6222*	*593.3148*	1168.5957	584.8015	1167.6117	584.3095	1169.6035	585.3054	1170.6113	585.8093	10
6	581.2678	291.1375	564.2413	282.6243	563.2572	282.1323	598.2943	299.6508	A		*1098.5902*	549.7987	1081.5637	541.2855	1080.5796	540.7935	1082.5715	541.7894	1083.5793	542.2933	9
7	678.3206	339.6639	661.2940	331.1506	660.3100	330.6586	695.3471	348.1772	P	984.5109	*1027.5531*	514.2802	*1010.5265*	505.7669	1009.5425	*505.2749*	1011.5344	506.2708	1012.5422	506.7747	8
8	*834.4217*	417.7145	817.3951	409.2012	816.4111	408.7092	851.4482	426.2278	R	828.4098	930.5003	465.7538	913.4738	457.2405	912.4898	456.7485	914.4816	457.7444	915.4894	458.2483	7
9	*963.4643*	482.2358	946.4377	473.7225	945.4537	473.2305	980.4908	490.7490	E		774.3992	387.7032	757.3727	379.1900	756.3886	378.6980	758.3805	379.6939	759.3883	380.1978	6
10	*1076.5483*	538.7778	1059.5218	530.2645	1058.5378	529.7725	1093.5749	547.2911	L		645.3566	323.1819	628.3301	314.6687	627.3461	314.1767	629.3379	315.1726	630.3457	315.6765	5
11	*1204.6433*	602.8253	*1187.6167*	*594.3120*	1186.6327	*593.8200*	1221.6698	611.3386	K		*532.2726*	266.6399	515.2460	*258.1266*	514.2620	*257.6346*	516.2538	*258.6306*	517.2617	259.1345	4
12	*1333.6859*	667.3466	*1316.6593*	658.8333	1315.6753	658.3413	1350.7124	675.8599	E		*404.1776*	202.5924	387.1510	194.0792	*386.1670*	193.5872	388.1589	*194.5831*	389.1667	*195.0870*	3
13	*1461.7445*	*731.3759*	*1444.7179*	722.8626	1443.7339	722.3706	1478.7710	739.8891	Q		275.1350	138.0711	*258.1084*	129.5579			259.1163	130.0618	260.1241	130.5657	2
14									Q		*147.0764*	74.0418	130.0499	65.5286			131.0577	66.0325	132.0655	66.5364	1

FIGURE 2 MS/MS fragmentation spectra of the (A) nondeamidated and (B) deamidated Asn408-Gln421 peptide in an overnight Arg-C digest of NPB-α2AP as determined by Mascot and MaxQuant analysis, respectively. Best spectrum data are shown. The overlapping labels of the peaks between m/z 730 and 750 in (A) should be read as the following: b(13)++ m/z 731.3759; y0(13)++,y*(13)++, z + 1(13)**, m/z 738.3837, 738.8757, 739.3796; y(13)++ m/z 747.3890

Mascot analysis data from the pilot experiment with Arg-C displayed one peptide (Asn408-Gln421) in the NPB-α2AP protein band that did not end with the Arg-C specific amino acid residue arginine (data not shown). This peptide was not present in PB-α2AP. Therefore, we hypothesized that this C-terminal end was already present in the purified α2AP sample and represents the in vivo cleavage site. The digestions with Asp-N, Lys-C, and trypsin did not result in any C-terminal ends that were not the result of protease specific cleavages. The experiment with Arg-C digestion of NPB-α2AP was repeated under optimized conditions to confirm the Gln421-Asp422 cleavage site. Mascot analysis data identified six peptides that could not be ascribed to the action of Arg-C: Asn408-Leu417, Asn408-Glu419, Asn408-Gln420, Asn408-Gln421, deamidated Asn408-Gln421, and Asn408-Asp422 (Table 2). MaxQuant analysis data from the optimized experiment revealed four of these six peptides: Asn408-Gln420, Asn408-Gln421, deamidated Asn408-Gln421, and Asn408-Asp422 (Table 2; Figures S1-S6). In both analysis methods, the Asn408-Gln421 peptide displayed the highest ions scores and best spectra (Figure 2). Therefore, our data suggest that the Gln421-Asp422 is a preferred cleavage site in the C-terminus of α2AP.

Taken together, our results suggest that in vivo NPB-α2AP results from cleavage after Leu417, Glu419, Gln420, Gln421, or Asp422, with Gln421 as preferred cleavage site (Figure 3). As expected, the Leu417-Asp422 region is located N-terminally from the TC 3AP epitope, explaining why NPB-α2AP is not detected by TC 3AP, as summarized in Figure 4.

4 | DISCUSSION

In this study, we determined the epitope of monoclonal antibody TC 3AP, which detects PB-α2AP but not in vivo formed NPB-α2AP.[20] Supported by this information, we specified where PB-α2AP is truncated in vivo for the generation of NPB-α2AP. We found that the TC 3AP epitope is located in a region between Asp428 and Gly439

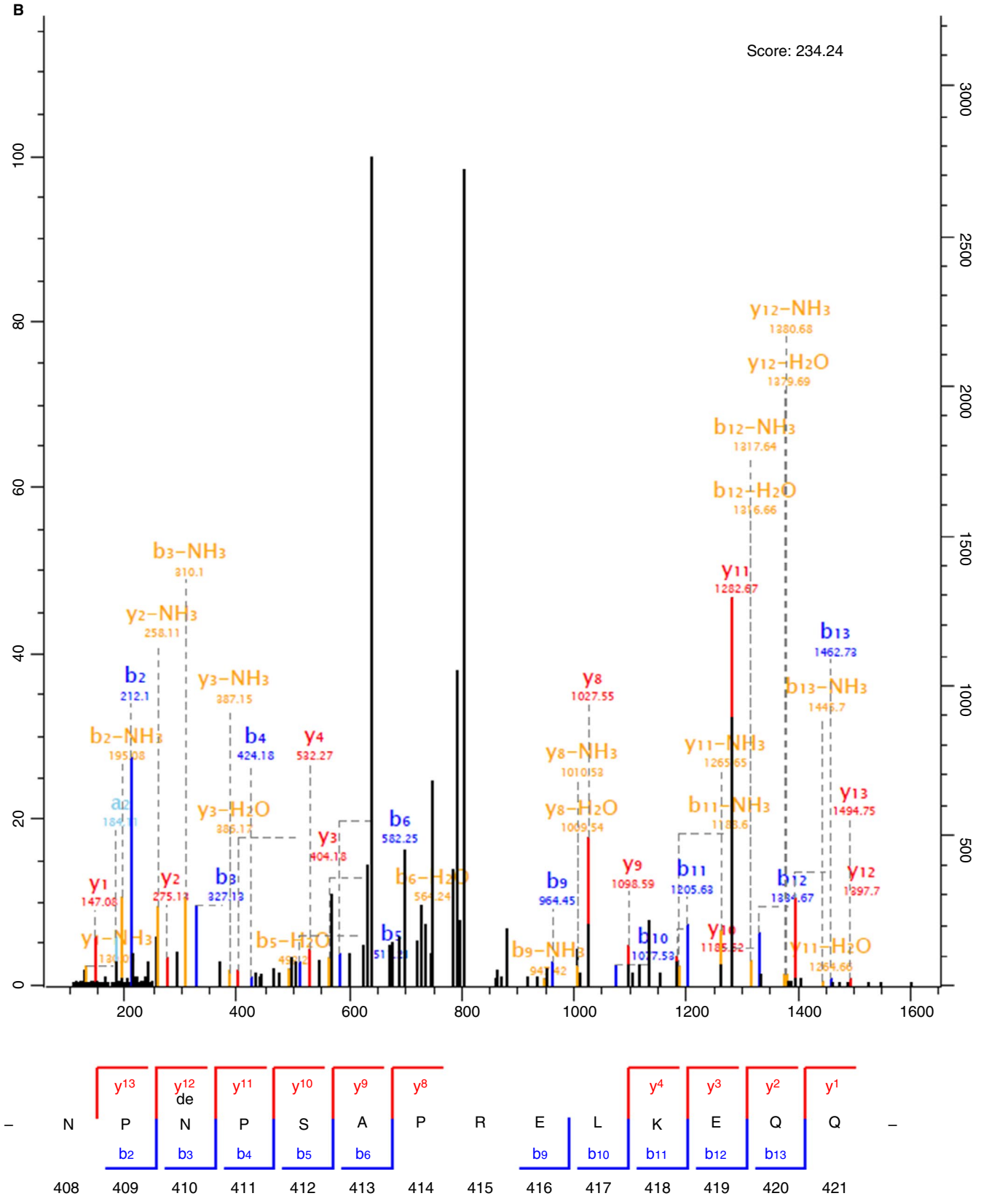


FIGURE 2 (Continued)

in the α2AP C-terminus and that NPB-α2AP results from multiple cleavages in PB-α2AP that occur in a region between Leu417 and Asp422.

The region of the TC 3AP epitope overlaps the region recognized by a commercial polyclonal antibody directed to α2AP (EB08777, Everest Biotech Ltd.). This commercial antibody was raised against

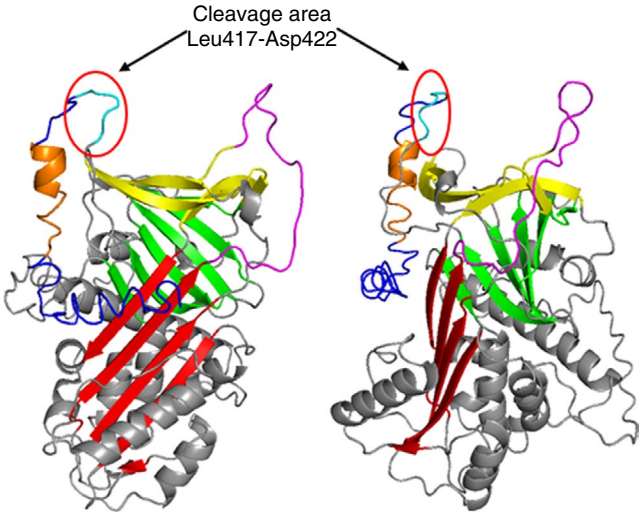


FIGURE 3 Three-dimensional structure of human α2AP as predicted by I-TASSER.[27] Red, β-sheet A; green, β-sheet B; yellow, β-sheet C; magenta, reactive center loop; cyan, C-terminal cleavage area Leu417-Asp422 (LKEQQD); blue, C-terminus; orange, epitope of TC 3AP. Left panel: front view. Right panel: side view, 90°C clockwise rotation

the peptide Lys427-Lys441. This suggests, that this C-terminal region is highly immunogenic and strengthens the reliability of our found TC 3AP epitope.

We found that in vivo cleavage of PB-α2AP occurs between Leu417 and Asp422. In a previous study, Clemmensen et al described the purification of PB-α2AP from plasma.[16] In that study, PB-α2AP (67 kDa) was spontaneously converted in vitro into two different forms with lower molecular weights (65 and 60 kDa). Native PB-α2AP was able to bind plasminogen and rapidly inactivate plasmin, whereas the 65-kDa form did not bind plasminogen but could still slowly inactivate plasmin. The 60-kDa form neither bound plasminogen nor inactivated plasmin. In our study, we focused on in vivo-generated NPB-α2AP. This NPB-α2AP form still contains the reactive site, which is needed for plasmin inhibition. Our MS data revealed several possible C-terminal cleavage sites, located C-terminally from the reactive site, indicating that the NPB-α2AP form still has inhibitory activity toward plasmin. Thus, although Sasaki et al showed that in vitro-generated 65 kDa NPB-α2AP lacks at most 26 amino acid residues (cleavage after Arg438),[17] our MS data show that in vivo-generated NPB-α2AP lacks about 43 amino acid residues by cleavage in a region between Leu417 and Asp422.

Leebeek et al[20] described that NPB-α2AP formed in vivo lost its capacity to bind to both plasminogen and TC 3AP, which is in line with our MS data showing that NPB-α2AP formed in vivo is cleaved in a region between Leu417 and Asp422, which is located N-terminally from the TC 3AP epitope. Furthermore, they showed that NPB-α2AP formed in vitro lost its plasminogen binding capacity, but could still bind to TC 3AP.[20] This in vitro-formed NPB-α2AP might be similar to the 65-kDa form of NPB-α2AP described by Sasaki et al,[17] which lacks at most 26 amino acid residues (Gly439-Lys464), and still contains the TC 3AP epitope located in a region between Asp428 and Gly439.

Five potential cleavage sites located in close proximity of each other were obtained from MS data, indicating the possibility of multiple NPB-α2AP forms and suggesting the presence of a proteolytic sensitive region (Figure 4). This makes the possibility of NPB-α2AP being the result of cleavage by one specific regulating protease unlikely. However, the highest ions scores and best spectra were found for the Asn408-Gln421 peptide. This peptide was also prominently found in the pilot experiment, suggesting that the Gln421-Asp422 is a preferred cleavage site. The Peptidase Database MEROPS (release 12.1) contains 33 enzymes capable of cleaving Gln-Asp bonds and might be helpful for finding candidate enzymes.

An alternative explanation for the five potential cleavage sites is that PB-α2AP is cleaved by one endopeptidase at Asp422-Ser423 and further truncated by different carboxypeptidases, such as thrombin-activatable fibrinolysis inhibitor and carboxypeptidase N. It is, however, unknown whether all required carboxypeptidases exist in plasma. Mutagenesis of α2AP could be useful to further study the cleavage region.

To date, there is no three-dimensional protein structure available for the complete C-terminus of α2AP, because of its flexibility.[26] Law et al[26] showed that the first 10 amino acids of the C-terminus of α2AP (Asn410-Glu419) are tightly associated with the serpin body, because residues 416 and 417 are incorporated in β-sheet C. The remainder of the C-terminus (Gln420-Lys464) could not be modeled into electron density. The C-terminal cleavage sites described in the current study are situated between Leu417 and Asp422, which are located in the beginning of the flexible C-terminus (Figure 3). Therefore, it is plausible that the found C-terminal cleavage sites are located in a region sensitive for proteolysis.

To conclude, we mapped the epitope of antibody TC 3AP and localized the in vivo C-terminal cleavage sites of PB-α2AP. These results can be used to gain more insight into the regulation of C-terminal heterogeneity of α2AP and thus the regulation of the fibrinolytic system.

FIGURE 4 Schematic representation of the α2AP C-terminus from Asn408 to Lys464. Arrows indicate the in vivo C-terminal cleavage sites (preferred site between Gln421 and Asp422), the bold underlined sequence represents the area that includes the TC 3AP epitope, and the brace indicates the area that includes the in vitro C-terminal cleavage site as described by Sasaki et al.[17]

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CONFLICT OF INTERESTS

The authors state that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

S. Abdul performed laboratory analyses, analyzed and interpreted data, and wrote the paper. D.H.W. Dekkers performed proteomic analyses and analyzed and interpreted data. R.A.S. Ariëns and F.W.G. Leebeek designed the research and interpreted data. D.C. Rijken and S. Uitte de Willige designed the research, interpreted data, and wrote the paper. All authors critically reviewed the manuscript and gave their consent to the final version.

ORCID

Dick H. W. Dekkers https://orcid.org/0000-0001-7057-6257

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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