Journal of Chromatography B, 911 (2012) 21–26

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Analysis of the extreme diversity of salivary alpha-amylase isoforms generated by physiological proteolysis using liquid chromatography–tandem mass spectrometry

Ulla-Maja Bailey[a,1], Chamindie Punyadeera[b,1], Justin J. Cooper-White[b], Benjamin L. Schulz[a,*]

[a] School of Chemistry & Molecular Biosciences, The University of Queensland, Brisbane, Queensland 4072, Australia
[b] Tissue Engineering and Microfluidic Laboratory, Saliva Translational Research Group, Australian Institute of Bioengineering and Nanotechnology, and School of Chemical Engineering, The University of Queensland, Brisbane, Queensland 4072, Australia

ARTICLE INFO

Article history:
Received 17 July 2012
Accepted 15 October 2012
Available online 26 October 2012

Keywords:
Saliva
Alpha-amylase
Liquid chromatography
Mass spectrometry
Proteolysis
Biomarker

ABSTRACT

Saliva is a crucial biofluid for oral health and is also of increasing importance as a non-invasive source of disease biomarkers. Salivary alpha-amylase is an abundant protein in saliva, and changes in amylase expression have been previously associated with a variety of diseases and conditions. Salivary alpha-amylase is subject to a high diversity of post-translational modifications, including physiological proteolysis in the oral cavity. Here we developed methodology for rapid sample preparation and non-targeted LC–ESI-MS/MS analysis of saliva from healthy subjects and observed an extreme diversity of alpha-amylase proteolytic isoforms. Our results emphasize the importance of consideration of post-translational events such as proteolysis in proteomic studies, biomarker discovery and validation, particularly in saliva.

1. Introduction

Saliva is an important biofluid in the oral cavity, aiding in physical lubrication, tooth enamel mineralisation, protection against infection, wound healing, mastication and taste sensation [1,2]. The components of whole saliva are contributed from various glandular secretions, including the parotid, submandibular and sublingual glands, gingival crevicular fluid, nasobronchial secretions, blood and plasma leakage from oral wounds, epithelial cells, food particles and microorganisms. Due to this high diversity of sources, the composition of whole saliva is complex and variable. Regulation of this variability is crucial for homeostasis of the oral cavity and hence in oral health. Alterations in the protein composition of saliva have also been associated with diverse diseases, including periodontitis [3], oral cancers [4], and autoimmune diseases such as Sjögrens Syndrome [5]. The changes in salivary protein composition observed in these states may contribute to the progression of these conditions, and may also be of use for identification of biomarkers for disease prognosis or diagnosis [2].

Salivary α-amylase is an abundant protein in saliva, with diverse roles in oral biology. α-Amylase catalyses the hydrolysis of internal α1-4 glucosidic bonds from various glucose polymers [6] and as such is an important enzyme in the early stages of carbohydrate digestion. Catalytically active salivary α-amylase has been reported to bind to microorganisms and tooth enamel, and as such is perhaps crucial in mediating adherence of pathogens to oral surfaces, and allowing microbial growth through provision of glucose from the breakdown of dietary glucose polymers [7–10]. Changes in the levels of salivary α-amylase have been reported in many proteomic studies, including implications in periodontitis [3], Sjögrens Syndrome [5,11], sensory perception of bitter tastes [12], and in binding of salivary tannins [13–16]. Salivary α-amylase is the product of the AMY1A, AMY1B and AMY1C genes, which encode identical protein sequences [17] but show inter-individual variation in copy number and hence α-amylase protein expression [18]. It has also been reported that α-amylase is subject to a very high level of posttranslational modification. The two potential N-glycosylation sites in salivary α-amylase are partially modified [19], and α-amylase is also subject to a high diversity of various post-translational modifications including deamidation of asparagine and glutamine residues [20]. In particular, protease digestion within the oral cavity results in a very large number of proteolytic isoforms of α-amylase. A previous study performed

* Corresponding author. Tel.: +61 7 3365 4875; fax: +61 7 3365 4273.
E-mail address: b.schulz@uq.edu.au (B.L. Schulz).
[1] These authors contributed equally to this work.

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.10.023

peptide fingerprint mapping with MALDI-TOF-MS of over 140 salivary amylase isoforms separated by 2D SDS-PAGE, confirming that this diversity is largely the result of proteolysis [21]. However, the relevance of this proteolytic diversity to proteomic analyses and biomarker discovery is unknown.

With the goal of examining the molecular diversity of salivary α-amylase, here we have developed and used a rapid and efficient combination of sample preparation and LC–ESI-MS/MS detection to perform a detailed non-targeted analysis of salivary α-amylase isoforms resulting from physiological proteolysis in the oral cavity. With the high sensitivity of this bottom-up proteomic approach we observe extreme diversity of proteolytic isoforms, map the sites of proteolysis and validate the diversity of selected proteolysis events in healthy subjects.

2. Materials and methods

2.1. Saliva collection

This study was approved by The University of Queensland Medical Ethical Institutional Board. All participants gave informed consent before sample collection. All study participants were over 18 years of age. Whole resting saliva was collected as described [22,23], supplemented with protease inhibitor cocktail and placed immediately on wet ice.

2.2. Sample preparation

Whole saliva samples were diluted directly to 6 M guanidinium chloride, 50 mM Tris–Cl pH 8 and 10 mM DTT, and incubated at 37 °C for 30 min. Cysteines were alkylated by addition of acrylamide to 25 mM and incubation at 23 °C for 1 h. Protein was subjected to methanol:acetone precipitation, resuspended in 50 mM ammonium acetate and digested with trypsin as described [24]. Peptides were desalted using C18 ZipTips (Millipore).

2.3. Mass spectrometry

Desalted peptides were analysed by LC–ESI-MS/MS using a Prominence nanoLC system (Shimadzu) and TripleTof 5600 mass spectrometer with a Nanospray III interface (ABSciex). Peptides were desalted on an Agilent C18 trap (5 μm, 0.3 mm × 5 mm) at a flow rate of 30 μl/min for 3 min, and then separated on a Vydac Everest C18 column (300 Å, 5 μm, 150 mm × 150 μm) at a flow rate of 1 μl/min. Peptides were separated with a gradient of 10–60% buffer B over 30 min, with buffer A (1% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile with 0.1% formic acid). Gas and voltage setting were adjusted as required. An MS TOF scan from m/z of 350–1800 was performed for 0.5 s followed by information dependent acquisition of MS/MS with automated CE selection of the top 20 peptides from m/z of 40–1800 for 0.05 s per spectrum.

2.4. Data analysis

Peptides were identified using an in-house installation of ProteinPilot, searching the UniProt database (as of 11th January 2012) with standard settings (Sample type, identification; cysteine alkylation, acrylamide; Instrument, TripleTOF 5600; Species, none; ID focus, biological modifications; Search effort, Thorough ID). False discovery rate analysis was performed for all samples. Peptides identified with greater than 99% confidence and with a local false discovery rate of less than 1% were included for further analysis, and MS/MS fragmentation spectra were manually inspected. Extracted ion chromatograms were obtained using PeakView 1.1. Protein structural analysis was performed using MacPymol.

3. Results and discussion

3.1. Identification of sites of physiological proteolysis in salivary α-amylase

We detected sites of physiological protease digestion of salivary α-amylase. We analysed trypsin-digested pooled whole saliva from healthy subjects by untargeted LC–ESI-MS/MS and identified peptides with ProteinPilot. This resulted in identification of 63 peptides from salivary α-amylase with over 99% confidence (Table 1). Remarkably, 2/3rds of these (43 independent peptides) were semi-tryptic peptides, representing 38 independent sites of physiological proteolysis.

3.2. Characteristics of salivary α-amylase proteolysis sites

To determine patterns behind the physiological proteolysis events we identified, we mapped these sites of proteolysis to the sequence of mature salivary α-amylase (Fig. 1A). This showed clusters of proteolysis, rather than an even spread of sites throughout the protein sequence. Further, we mapped the sites of physiological proteolysis on the 3D crystal structure of salivary α-amylase [25] (Fig. 1B–D). Again, this showed clustering of sites of proteolysis, with almost all proteolysis events mapping to surface exposed portions of the α-amylase protein. While proteolysis sites generally mapped to the surface of α-amylase, they were not evenly distributed over the protein surface. Intriguingly, sites of proteolysis mapped to regions of α-amylase previously reported to be not involved in non-catalytic binding of glucose polysaccharides [26]. This suggests that binding of saccharide to portions of the surface of α-amylase in saliva in the oral cavity provides physical protection of the underlying protein surface, while the remaining exposed α-amylase surface is accessible to protease digestion.

3.3. Non-surface exposed salivary α-amylase proteolysis sites

Although most clusters of physiological proteolysis were on the surface of the mature α-amylase protein, some sites were not surface exposed. One such cluster of proteolysis sites was from M323 to M328 (Table 1 and Fig. 2). These residues are in the middle section of an α-helix on the side of the core α-amylase catalytic domain, but covered by the 90 amino acid C-terminal domain of α-amylase. Several physiological proteolysis sites mapped to this C-terminal domain, including at F406 and N408, present in a short linker between the core and C-terminal domains. This suggests that cleavage at F406 or N408 may release the C-terminal domain, expose this side of the core α-amylase domain and allow subsequent proteolysis at the M323-M328 cluster.

3.4. Abundance of salivary α-amylase physiological proteolysis events

The intensity of extracted ion chromatograms corresponding to tryptic and semi-tryptic peptides from the M323 to M328 proteolysis cluster suggest that the majority of α-amylase is not subject to proteolysis at this site, as the semi-tryptic peptides show much lower intensity than the non-proteolytic full-length tryptic peptide (Fig. 2 and Table 1). This pattern was also present in comparison of the intensity of all tryptic and semi-tryptic peptides, with full tryptic peptides being on average approximately ten fold more intense than semi-tryptic peptides (Fig. 3A and Table 1). This suggests that the detected physiological proteolysis sites are individually rare in the overall pool of salivary α-amylase proteins. However, given the extreme diversity of potential proteolysis sites we identified, it is in fact likely that the majority of α-amylase proteins are

Table 1
Tryptic and semi-tryptic peptides from salivary α-amylase.

Position	Sequence	Semi-tryptic cleavages	*m/z*	*z*	Δmass	Precursor signal
11–20	TSIVHLFEWR		644.35	2	0.0000	1.03E+06
12–20	SIVHLFEWR	T-S@N-term	593.82	2	−0.0005	1.06E+05
13–20	IVHLFEWR	S-I@N-term	550.31	2	−0.0009	2.77E+04
21–30	WVDIALECER		652.82	2	−0.0001	1.13E+05
36–56	GFGGVQVSPPNENVAIHNPFR	R-P@C-term	1118.57	2	0.0027	1.59E+04
36–61	GFGGVQVSPPNENVAIHNPFRPWWER		997.50	3	0.0010	8.90E+05
43–61	SPPNENVAIHNPFRPWWER	V-S@N-term	1173.58	2	−0.0001	5.38E+03
44–61	PPNENVAIHNPFRPWWER	S-P@N-term	753.71	3	0.0012	4.89E+04
45–61	PNENVAIHNPFRPWWER	P-P@N-term	1081.54	2	0.0018	2.10E+04
49–61	VAIHNPFRPWWER	N-V@N-term	427.73	4	0.0030	4.83E+04
93–102	IYVDAVINHM	M-C@C-term	587.80	2	0.0006	8.11E+04
93–105	IYVDAVINHMCGN	N-A@C-term	760.35	2	−0.0028	2.75E+04
93–124	IYVDAVINHMCGNAVSAGTSSTCGSYFNPGSR		1140.85	3	0.0039	4.12E+04
103–124	CGNAVSAGTSSTCGSYFNPGSR	M-C@N-term	1132.99	2	−0.0033	4.62E+03
106–124	AVSAGTSSTCGSYFNPGSR	N-A@N-term	960.43	2	−0.0021	2.38E+04
125–131	DFPAVPY	Y-S@C-term	404.70	2	0.0008	1.48E+04
125–140	DFPAVPYSGWDFNDGK		907.91	2	0.0020	2.37E+06
143–158	TGSGDIENYNDATQVR		870.39	2	−0.0007	1.08E+05
162–176	LSGLLDLALGKDYVR		544.98	3	−0.0002	2.54E+05
163–176	SGLLDLALGKDYVR	L-S@N-term	760.42	2	−0.0060	1.78E+04
164–176	GLLDLALGKDYVR	S-G@N-term	716.91	2	−0.0017	1.27E+04
179–195	IAEYMNHLIDIGVAGFR		640.33	3	−0.0006	1.87E+06
180–195	AEYMNHLIDIGVAGFR	I-A@N-term	903.45	2	0.0021	5.22E+04
181–195	EYMNHLIDIGVAGFR	A-E@N-term	867.93	2	−0.0016	4.02E+04
182–195	YMNHLIDIGVAGFR	E-Y@N-term	803.41	2	−0.0021	1.52E+04
183–195	MNHLIDIGVAGFR	Y-M@N-term	481.59	3	0.0015	5.41E+03
185–195	HLIDIGVAGFR	N-H@N-term	399.90	3	0.0012	1.39E+04
201–208	HMWPGDIK		492.24	2	−0.0021	1.27E+05
214–227	LHNLNSNWFPEGSK	K-P@C-term	821.90	2	0.0009	2.36E+05
214–229	LHNLNSNWFPEGSKPF	F-I@C-term	629.65	3	0.0031	1.88E+05
214–234	LHNLNSNWFPEGSKPFIYQEV	V-I@C-term	840.42	3	−0.0009	1.56E+05
214–243	LHNLNSNWFPEGSKPFIYQEVIDLGGEPIK		1147.92	3	0.0014	3.55E+05
219–243	SNWFPEGSKPFIYQEVIDLGGEPIK	N-S@N-term	950.82	3	0.0032	2.08E+04
223–243	PEGSKPFIYQEVIDLGGEPIK	F-P@N-term	1158.61	2	−0.0054	1.39E+04
228–243	PFIYQEVIDLGGEPIK	K-P@N-term	909.49	2	0.0010	5.45E+05
230–243	IYQEVIDLGGEPIK	F-I@N-term	787.43	2	0.0005	1.56E+05
244–252	SSDYFGNGR		501.72	2	−0.0011	1.18E+04
279–291	NWGEGWGFMPSDR		769.83	2	−0.0010	2.44E+06
292–303	ALVFVDNHDNQR		714.35	2	−0.0078	7.03E+04
294–303	VFVDNHDNQR	L-V@N-term	622.29	2	−0.0012	2.73E+04
304–315	GHGAGGASILTF	F-W@C-term	544.28	2	0.0012	1.68E+04
304–319	GHGAGGASILTFWDAR		808.40	2	0.0006	1.01E+06
323–333	MAVGFMLAHPY	Y-G@C-term	618.80	2	−0.0002	3.19E+04
323–335	MAVGFMLAHPYGF	F-T@C-term	720.84	2	−0.0029	3.00E+05
323–337	MAVGFMLAHPYGFTR		849.42	2	−0.0003	6.91E+05
324–337	AVGFMLAHPYGFTR	M-A@N-term	783.90	2	−0.0008	4.73E+04
325–337	VGFMLAHPYGFTR	A-V@N-term	748.38	2	0.0000	9.39E+04
326–337	GFMLAHPYGFTR	V-G@N-term	698.85	2	0.0000	5.45E+04
327–337	FMLAHPYGFTR	G-F@N-term	670.33	2	−0.0032	2.07E+04
328–337	MLAHPYGFTR	F-M@N-term	596.80	2	−0.0032	3.94E+04
347–368	YFENGKDVNDWVGPPNDNGVTK		1233.07	2	−0.0004	1.12E+05
353–368	DVNDWVGPPNDNGVTK		576.27	3	−0.0038	1.42E+05
369–387	EVTINPDTTCGNDWVCEHR		1166.01	2	−0.0014	4.45E+04
373–387	NPDTTCGNDWVCEHR	I-N@N-term	944.89	2	−0.0010	3.14E+03
399–421	NVVDGQPFTNWYDNGSNQVAFGR		862.40	3	−0.0032	4.39E+05
407–421	TNWYDNGSNQVAFGR	F-T@N-term	865.38	2	0.0009	1.05E+05
409–421	WYDN[a]GSNQVAFGR	N-W@N-term	757.35	2	0.0020	2.68E+04
475–495	AHFSISNSAEDPFIAIHAESK		757.71	3	−0.0002	5.79E+05
478–495	SISNSAEDPFIAIHAESK	F-S@N-term	958.47	2	−0.0028	1.42E+04
482–495	SAEDPFIAIHAESK	N-S@N-term	505.59	3	−0.0023	2.18E+04
483–495	AEDPFIAIHAESK	S-A@N-term	714.36	2	−0.0013	1.92E+04
484–495	EDPFIAIHAESK	A-E@N-term	678.84	2	0.0018	4.21E+04
485–495	DPFIAIHAESK	E-D@N-term	614.32	2	0.0006	1.75E+04

[a] Deamidated Asn. All Cys are alkylated. The most confidently identified form of each peptide is reported.

subject to physiological proteolysis, in agreement with previous proteomic studies [21]. It is possible that initial proteolysis may be rate limiting, and the flexibility engendered by this cleavage leads to an increased rate of subsequent proteolysis at nearby or distal sites. It is also conceivable that many of the proteolytically processed α-amylase isoforms may still be functional. Five disulfide bonds are present in the mature structure of salivary α-amylase [25], and as structural disulfide bonds these serve to increase the stability of the mature protein. Proteolytic digest of a single surface site would likely increase local flexibility, but with the overall protein structure retained. The influence on the glucosidic hydrolysis or saccharide binding activities of α-amylase could be variable depending on the site and number of proteolysis events in a given α-amylase protein molecule.

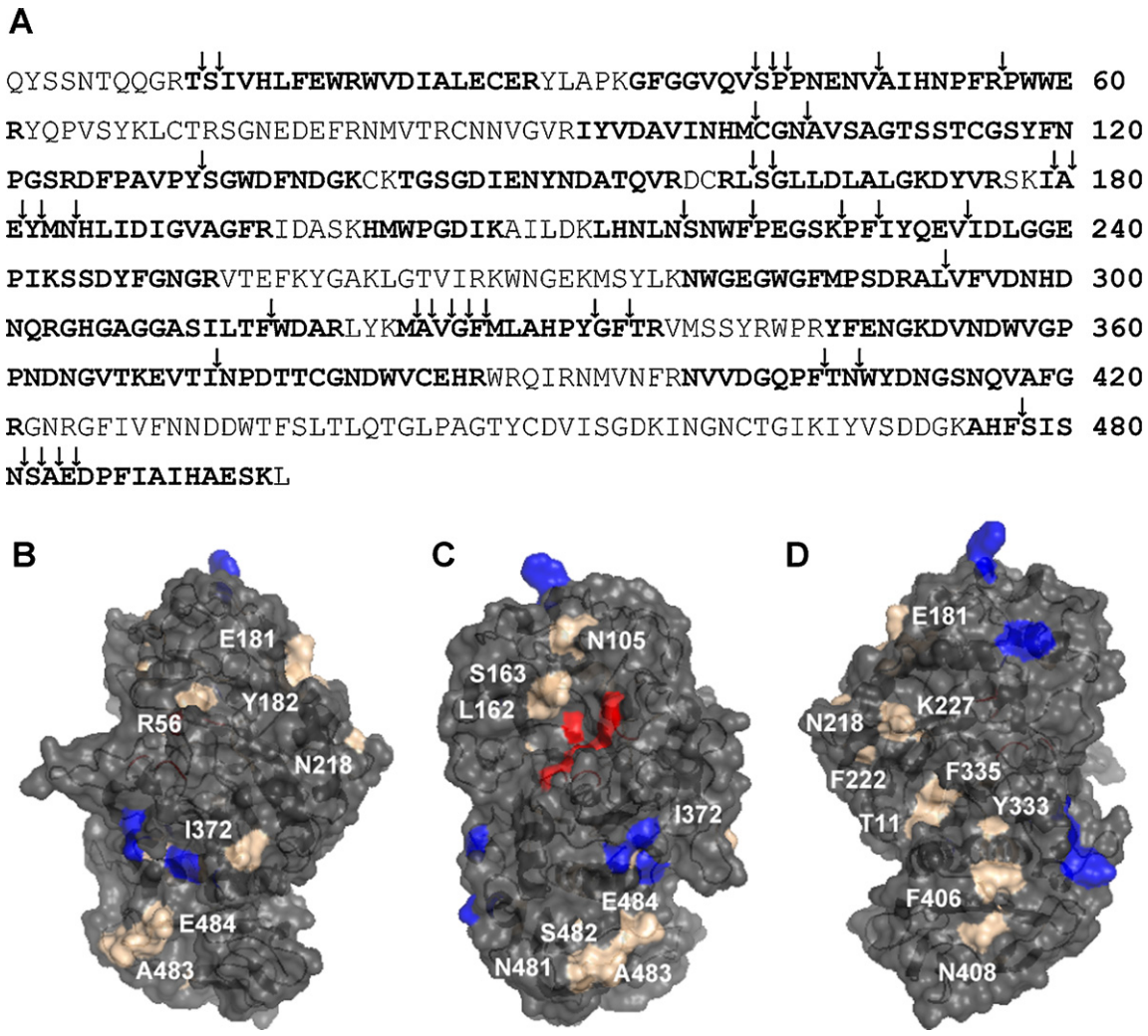

Fig. 1. Sites of physiologic proteolysis in salivary α-amylase. (A) Sequence of mature salivary α-amylase. Sequence coverage by tryptic and semi-tryptic peptides (Table 1) is shown in bold. Arrows show detected sites of physiologic proteolysis as determined by the non-tryptic termini of semi-tryptic peptides. (B–D) Rotated views of surface representations of the crystal structure of salivary α-amylase [25]. Sites of previously reported non-catalytic saccharide binding [26] are coloured blue. Selected sites of physiological proteolysis (Table 1) are labelled and coloured wheat. Active site residues are coloured red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Salivary α-amylase proteolytic diversity

The previous most detailed study on proteolytic isoforms of salivary α-amylase reported approximately 140 isoforms detectable by 2D SDS-PAGE [21]. This previous study used MALDI-TOF-MS and peptide mass fingerprinting with predicted full tryptic peptides to map the coverage of amylase isoforms. The use of full tryptic peptides limited the resolution at which the proteolysis events resulting in isoforms could be mapped to the closest lysine or arginine residue. In contrast, our analysis here has mapped the precise sites of proteolysis with untargeted LC–ESI-MS/MS detection and database searching, including identification of semi-tryptic peptides which precisely map the sites of physiological proteolysis. In general, our approach detected that sites of proteolysis were clustered within the α-amylase sequence (Table 1, Figs. 1 and 2). This suggests that many of the individual 2D SDS-PAGE gel spots identified as salivary α-amylase isoforms [21] actually contain multiple isoforms, differing only by a few amino acids. In turn, this implies that the actual physiological proteolytic isoform diversity of salivary α-amylase is much greater than these ~140 spots. As the clusters of proteolysis we detected consisted of an average of 3.3 independent precise sites of proteolysis, the total number of physiological proteolytic salivary amylase isoforms is likely more than triple the number of 2D SDS-PAGE spots, or more than 500 distinct isoforms. The presence of any additional rare, low abundant proteolysis sites would further increase the diversity of salivary α-amylase isoforms. Additionally, very small proteins may not have been efficiently precipitated by acetone:methanol treatment, potentially further increasing isoform diversity. The bottom-up proteomic analysis we report here has high sensitivity but results in loss of connectivity information between peptides. Top-down proteomic approaches would be required to further elucidate the precise α-amylase isoforms present in saliva.

3.6. Validation of salivary α-amylase proteolysis

To gauge the generality of our findings, we validated a selected subset of proteolysis events by LC–ESI-MS/MS analysis of individual whole saliva samples from healthy subjects. For this we used the proteolysis cluster internal to mature α-amylase, M323–M328 (Fig. 2), and determined the intensity of full-length tryptic and semi-tryptic peptides resulting from physiological proteolysis as judged by extracted ion chromatograms in these individuals (Fig. 3B). The same semi-tryptic peptides were detected in all

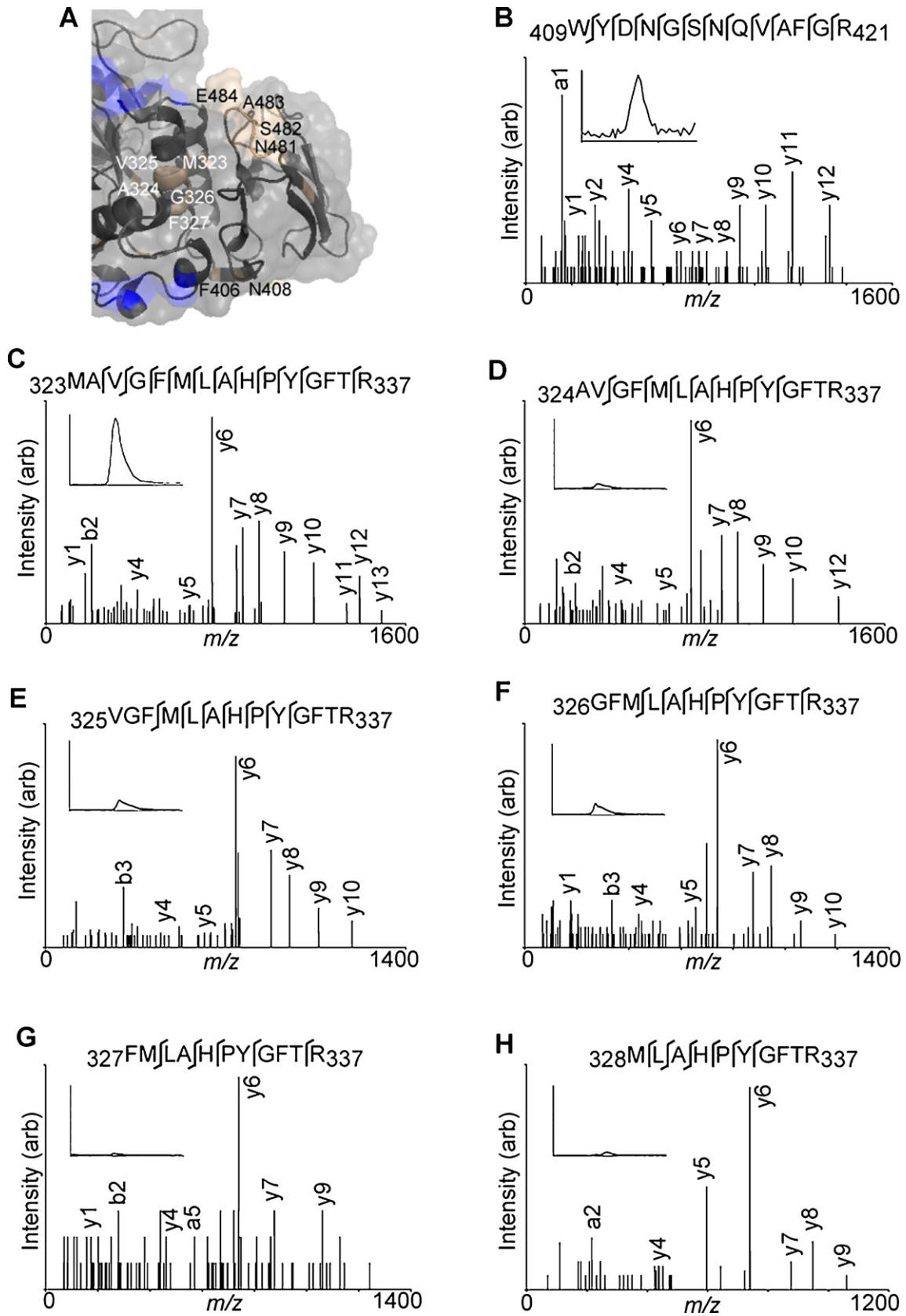

Fig. 2. Physiological proteolysis of an internal section of salivary α-amylase. (A) Cartoon of mature salivary α-amylase [25]. Sites of physiological proteolysis (Table 1) are labelled and coloured wheat. MS/MS spectra identifying full-length tryptic (B and C) and semi-tryptic (D–H) peptides from this region, with corresponding extracted ion chromatograms as inserts (MS/MS spectra and extracted ion chromatograms from ions with *m/z* of: (B) 757.35^{2+}, (C) 849.42^{2+}, (D) 783.90^{2+}, (E) 748.38^{2+}, (F) 698.85^{2+}, (G) 670.33^{2+}, (H) 596.80^{2+}; see Table 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

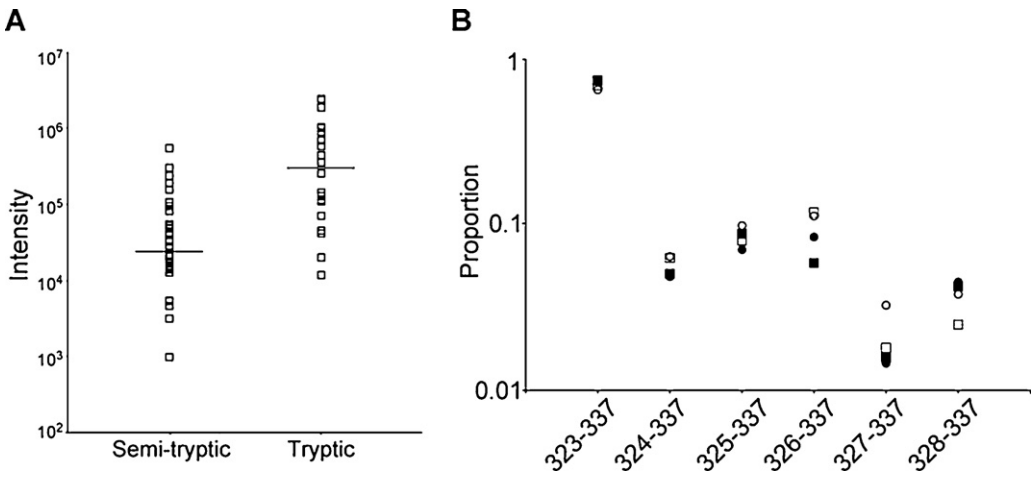

Fig. 3. (A) Intensity of tryptic and semi-tryptic peptides from salivary α-amylase. Horizontal bars show mean. (B) Validation of selected proteolytic cleavage events in healthy subjects. Proportion of intensity of tryptic and semi-tryptic peptides corresponding to proteolysis at sequential positions between M323 and L329. Data points (■, □, ●, ○) represent different individuals.

samples, suggesting that these physiological proteolysis events are common in healthy subjects. Further, the relative abundances of each of these tryptic and semi-tryptic peptides showed limited variability between individuals. This supports the utility of the rapid LC–ESI-MS/MS analysis we performed for semi-quantitative analysis of multiple sites of physiological proteolysis in salivary α-amylase.

4. Conclusions

In conclusion, we have developed and used a rapid and straightforward method for untargeted LC–ESI-MS/MS analyses of trypsin-digested proteins from minimally processed whole saliva to map the sites of physiological proteolysis of salivary α-amylase. Our results emphasize the unusually high extent of modification of this abundant salivary protein, which is of direct relevance to the discovery, validation and use of α-amylase and other proteins as disease biomarkers in saliva.

Acknowledgements

Financial support for this work was provided by NHMRC project grant 631615 and Career Development Fellowship APP1031542 to BLS, and Queensland Government Smart Futures Fellowship and The University of Queensland Foundation Research Excellence Award to CP.

References

[1] T. Pfaffe, J. Cooper-White, P. Beyerlein, K. Kostner, C. Punyadeera, Clin. Chem. 57 (2011) 675.
[2] B.L. Schulz, J.C. White, C. Punyadeera, Crit. Rev. Biotechnol. (2012), http://dx.doi.org/10.3109/07388551.2012.687361
[3] Y. Wu, R. Shu, L.J. Luo, L.H. Ge, Y.F. Xie, J. Periodontal Res. 44 (2009) 636.
[4] P. Dowling, R. Wormald, P. Meleady, M. Henry, A. Curran, M. Clynes, J. Proteomics 71 (2008) 168.
[5] C. Baldini, L. Giusti, F. Ciregia, Y. Da Valle, C. Giacomelli, E. Donadio, F. Semissi, L. Bazzichi, G. Giannaccini, S. Bombardieri, A. Lucacchini, Arthritis Res. Ther. 13 (2011) R197.
[6] A.D. Merritt, R.C. Karn, Adv. Hum. Genet. 8 (1977) 135.
[7] S. Choi, E. BJ, J.H. Jeon, K. Cho, D.G. Seo, K.Y. Kum, C.H. Yun, S.H. Han, Mol. Immunol. 48 (2011) 2207.
[8] L.S. Bruno, X. Li, L. Wang, R.V. Soares, C.C. Siqueira, F.G. Oppenheim, R.F. Troxler, G.D. Offner, Biochim. Biophys. Acta 1746 (2005) 65.
[9] I. Iontcheva, F.G. Oppenheim, R.F. Troxler, J. Dent. Res. 76 (1997) 734.
[10] W.L. Siqueira, F.G. Oppenheim, Arch. Oral Biol. 54 (2009) 437.
[11] Y. Fleissig, O. Deutsch, E. Reichenberg, M. Redlich, B. Zaks, A. Palmon, D.J. Aframian, Oral Dis. 15 (2009) 61.
[12] M. Dsamou, O. Palicki, C. Septier, C. Chabanet, G. Lucchi, P. Ducoroy, M.C. Chagnon, M. Morzel, Chem. Senses 37 (2012) 87.
[13] G. da Costa, E. Lamy, F. Capela e Silva, J. Andersen, E. Sales Baptista, A.V. Coelho, J. Chem. Ecol. 34 (2008) 376.
[14] K. Hara, M. Ohara, I. Hayashi, T. Hino, R. Nishimura, Y. Iwasaki, T. Ogawa, Y. Ohyama, M. Sugiyama, H. Amano, Eur. J. Oral Sci. 120 (2012) 132.
[15] A. Zajacz, G. Gyemant, N. Vittori, L. Kandra, Carbohydr. Res. 342 (2007) 717.
[16] E. Lamy, G. Graca, G. da Costa, C. Franco, F.C.E. Silva, E.S. Baptista, A.V. Coelho, Proteome Sci. 15 (2010) 65.
[17] D.L. Gumucio, K. Wiebauer, R.M. Caldwell, C. Samuelson, M.H. Meisler, Mol. Cell. Biol. 8 (1988) 1197.
[18] A.L. Mandel, C. Peyrot des Gachons, K.L. Plank, S. Alarcon, P.A. Breslin, PLoS One 5 (2010) e13352.
[19] P. Ramachandran, P. Boontheung, Y. Xie, M. Sondej, D.T. Wong, J.A. Loo, J. Proteome Res. 5 (2006) 1493.
[20] R.C. Karn, J.D. Shulkin, A.D. Merritt, R.C. Newell, Biochem. Genet. 10 (1973) 341.
[21] C. Hirtz, F. Chevalier, D. Centeno, V. Rofidal, J.C. Egea, M. Rossignol, N. Sommerer, D. Deville de Periere, Proteomics 5 (2005) 4597.
[22] C. Punyadeera, G. Dimeski, K. Kostner, P. Beyerlein, J. Cooper-White, J. Immunol. Methods 373 (2011) 19.
[23] E. Topkas, P. Keith, G. Dimeski, J. Cooper-White, C. Punyadeera, Clin. Chim. Acta 413 (2012) 1066.
[24] M.F.B. Jamaluddin, U.M. Bailey, N.Y.J. Tan, A.P. Stark, B.L. Schulz, Protein Sci. 20 (2011) 849.
[25] N. Ramasubbu, V. Paloth, Y. Luo, G.D. Brayer, M.J. Levine, Acta Crystallogr. D: Biol. Crystallogr. 52 (1996) 435.
[26] C. Ragunath, S.G. Manuel, V. Venkataraman, H.B. Sait, C. Kasinathan, N. Ramasubbu, J. Mol. Biol. 384 (2008) 1232.