Supplementary Information

Millimeter Water-in-Oil Droplet as an Alternative Back Exchange Prevention Strategy for Hydrogen/Deuterium Exchange Mass Spectrometry of Peptide/Protein

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Evaluation of the In-Source Artificial Back Exchange Using Maltoheptose

The artificial back exchange extent in LMJ-SSP-MS is evaluated using carbohydrate containing only fast-exchanging hydroxyl group. Maltoheptose is reported to be a suitable internal standard for evaluating the artificial in-source back exchange occurred during ESI[1], and it is used for LMJ-SSP-MS. We didn't apply maltoheptose as an internal standard in our experiments, since we are monitoring the back exchange prior to LMJ-SSP-MS analysis and back exchange occur before and during LMJ-SSP-MS analysis cannot be distinguished. For instance, the experimental workflow used in the subsection "Decelerated Back Exchange in Water-in-oil Droplet" involves a 2-min enzymatic digestion of fully deuterated Mb in protiated pepsin solution with a protein-to-pepsin volume ratio of 1-to-9. Since the D/H volume ratio of the reaction mixture was 1-to-9, the deuterium labels on the fully deuterated maltoheptose would probably lose readily once spiked into the reaction mixture. Therefore, the evaluation of back exchange in the subsequent LMJ-SSP-MS analysis would not be possible.

To solve this problem, we used maltoheptose in a separate experiment which the undeuterated Mb in H_2O was digested with pepsin solution prepared in D_2O using a protein-to-pepsin volume ratio of 1-to-9. The final D/H volume ratio of the resultant mixture was thereby 9-to-1. Practically, 0.5 μL of 1mM maltoheptose was spiked into 25 μL of the Mb/pepsin mixture containing 90% (v/v) D_2O and experienced the 2-min digestion process with Mb. 25 μL of ACN was then added to quench the digestion. Finally, 1 μL of the solution was added into the cyclohexane reservoir preloaded in the cut Eppendorf, followed by an LMJ-SSP-MS analysis of the water-in-oil droplet. All the instrumental parameters used for the analysis, including the spraying solvent composition and the spraying solvent flow rate, were the same as described in the main manuscript.

The resultant deuterium uptake of maltoheptose obtained by the LMJ-SSP-MS analysis was quantified to be 20.52 Da (±0.58) (Data not shown). Maltoheptose contains 23 fast-exchanging hydroxyl protons, the deuterium uptake of 20.52 Da signify an 89% deuteration. Since the hydroxyl would equilibrate to the percentage of deuterium in the surrounding solution, the maximum deuteration percentage of maltoheptose would be 90%. The 89% deuteration recorded by LMJ-SSP-MS almost reached 90%, indicating a negligible deuterium loss during the LMJ-SSP-MS analysis. Even the deuterium labels on fast-exchanging hydroxyl protons can be well-preserved, the back exchange of the backbone amide hydrogens which possess a much slower H/D exchange rate under acidic conditions could be deduced to be negligible for our LMJ-SSP-MS platform.

Extraction of Deuterium Level Information from MS Spectra

The procedures for extracting deuterium level information from MS spectra is illustrated using the peptide fragment 56-69. Figure S1A displayed the isotopic clusters of the 56-69 fragment ions (+3) obtained from (A) undeuterated Mb digest mixture drop-casted on Teflon surface; (B) deuterated Mb digest mixture drop-casted on Teflon surface; (C) deuterated Mb digest mixture drop-casted and completely dried on Teflon surface; and (D) deuterated Mb digest mixture stored in the form of water-in-oil droplet for 20 mins.

As computed using *HX Express*[2], the centroid masses of the isotopic clusters showed in Figure S1A, S1B, S1C and S1D are 1524.77 Da, 1531.34 Da, 1525.11 Da and 1528.14 Da respectively. Deducting the centroid mass of the undeuterated peptide (Figure S1A) from the deuterated peptide yielded the deuterium uptake value of 6.57 Da, 0.34 Da and 3.37 Da for Figure S1B, S1C and S1D respectively. The same deuterium level quantification procedures were applied to the MS raw data obtained from the three experimental runs, and the resultant average deuterium level were plotted in Figure 3 of the main manuscript.

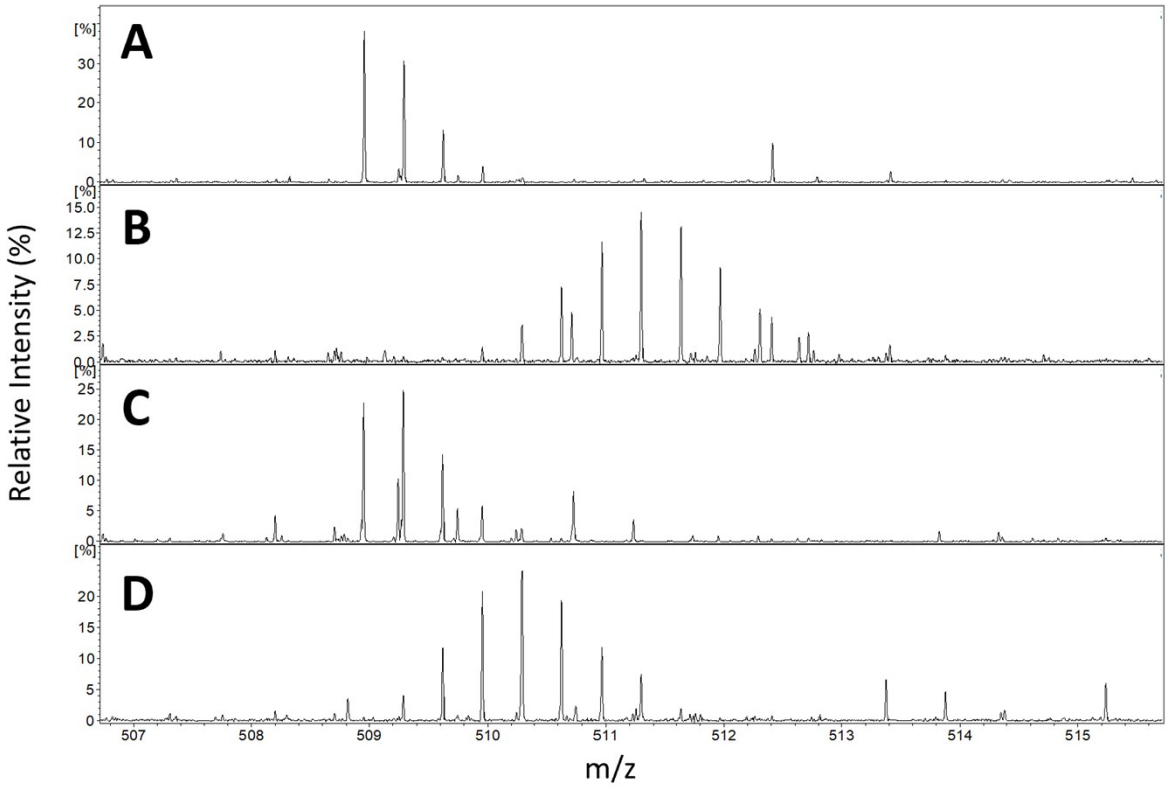

Figure S1. Representative mass spectra showing the isotopic clusters of the 56-69 fragment ions (+3). Representative mass spectra showing the isotopic clusters of the 56-69 fragment ions (+3) obtained from (A) undeuterated Mb digest mixture drop-casted on Teflon surface; (B) deuterated Mb digest mixture drop-casted on Teflon surface; (C) deuterated Mb digest mixture drop-casted and completely dried on Teflon surface; and (D) deuterated Mb digest mixture stored in the form of water-in-oil droplet for 20 mins.

Numbers and Rate Constants of Fast, Intermediate, and Slow Exchanging Backbone Amide Hydrogens Involved in D-to-H Back Exchange Reaction

Fragment 30-55		Bulk	Water-in-Oil Droplet
Fast exchanging	**Rate Constant (k_1, min^{-1})**	0.346	0.091
	Number (N_1)	4.516	5.542
Intermediate Exchanging	**Rate Constant (k_2, min^{-1})**	0.236	0.091
	Number (N_2)	4.228	4.632
Slow Exchanging	**Rate Constant (k_3, min^{-1})**	0.022	0.000
	Number (N_3)	5.878	4.556

Table S1. Numbers and Rate Constants of Backbone Amide Hydrogens Involved in Back Exchange Reaction for Fragment 30-55. Numbers and rate constants of fast, intermediate and slow exchanging backbone amide hydrogens involved in the D-to-H back exchange reaction for fragment 30-55 are obtained using MS Excel Solver. The back exchange rate constants for all groups of backbone amide hydrogens are smaller in water-in-oil droplet than that in bulk solution, implying a decelerated back exchange reaction in water-in-oil droplet than bulk solution.

Fragment 70-106		Bulk	Water-in-Oil Droplet
Fast exchanging	**Rate Constant (k_1, min^{-1})**	0.231	0.075
	Number (N_1)	11.273	12.888
Intermediate Exchanging	**Rate Constant (k_2, min^{-1})**	0.000	0.000
	Number (N_2)	8.449	5.750
Slow Exchanging	**Rate Constant (k_3, min^{-1})**	0.000	0.000
	Number (N_3)	0.411	1.541

Table S2. Numbers and Rate Constants of Backbone Amide Hydrogens Involved in Back Exchange Reaction for Fragment 70-106. Numbers and rate constants of fast, intermediate and slow exchanging backbone amide hydrogens involved in the D-to-H back exchange reaction for fragment 70-106 are obtained using MS Excel Solver. The back exchange rate constants for fast exchanging backbone amide hydrogens are smaller in water-in-oil droplet than that in bulk solution, implying a decelerated back exchange reaction in water-in-oil droplet than bulk solution. Meanwhile, the rate constants for intermediate and slow exchanging backbone amide in both reaction environments approaches zero, implying

these backbone amide hydrogens did not undergo back exchange reaction in the 10 mins experiment time course.

Fragment 110-134		**Bulk**	**Water-in-Oil Droplet**
Fast exchanging	**Rate Constant (k_1, min^{-1})**	0.609	0.137
	Number (N_1)	4.026	0.465
Intermediate Exchanging	**Rate Constant (k_2, min^{-1})**	0.047	0.131
	Number (N_2)	9.623	6.477
Slow Exchanging	**Rate Constant (k_3, min^{-1})**	0.000	0.005
	Number (N_3)	0.434	7.059

Table S3. Numbers and Rate Constants of Backbone Amide Hydrogens Involved in Back Exchange Reaction for Fragment 110-134. Numbers and rate constants of fast, intermediate and slow exchanging backbone amide hydrogens involved in the D-to-H back exchange reaction for fragment 110-134 are obtained using MS Excel Solver. The back exchange rate constants for fast exchanging backbone amide hydrogens are smaller in water-in-oil droplet than that in bulk solution, implying a decelerated back exchange reaction in water-in-oil droplet than bulk solution. Meanwhile, the rate constants for both intermediate and slow exchanging backbone amide in water-in-oil droplet is larger than that in bulk solution. However, a greater number of backbone amide hydrogen is categorized as intermediate or slow exchanging group, as well as a smaller number of backbone amide hydrogens are categorized into the fast exchanging group in water-in-oil droplet than in bulk reaction. The overall apparent effect is still the deceleration of back exchange reaction in the water-in-oil droplet.

Fragment 138-153		**Bulk**	**Water-in-Oil Droplet**
Fast exchanging	**Rate Constant (k_1, min^{-1})**	0.397	0.138
	Number (N_1)	2.313	4.722
Intermediate Exchanging	**Rate Constant (k_2, min^{-1})**	0.030	0.000
	Number (N_2)	8.619	5.581
Slow Exchanging	**Rate Constant (k_3, min^{-1})**	0.000	0.000
	Number (N_3)	0.407	1.056

Table S4. Numbers and Rate Constants of Backbone Amide Hydrogens Involved in Back Exchange Reaction for Fragment 138-153. Numbers and rate constants of fast, intermediate and slow exchanging backbone amide hydrogens involved in the D-to-H back

exchange reaction for fragment 138-153 are obtained using MS Excel Solver. The back exchange rate constants for fast and intermediate exchanging backbone amide hydrogens are smaller in water-in-oil droplet than that in bulk solution, implying a decelerated back exchange reaction in water-in-oil droplet than bulk solution. Meanwhile, the rate constants for slow exchanging backbone amide in both reaction environments approaches zero, implying these backbone amide hydrogens did not undergo back exchange reaction in the 10 mins experiment time course.

Reaction Mechanism of Acid-Catalyzed Back Exchange Reaction of Deuterated Backbone Amide

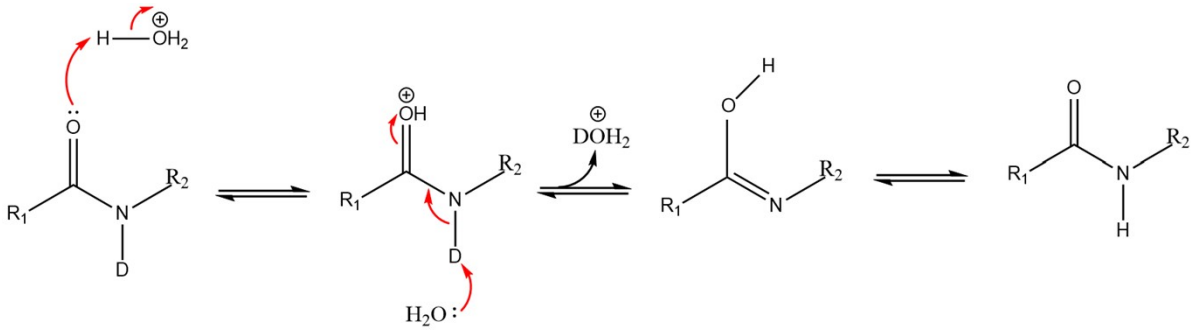

Figure S2. Reaction mechanism of acid-catalyzed back exchange reaction of deuterated backbone amide. Reaction mechanism of acid-catalysed back exchange reaction of deuterated backbone amide, in which both the first step i.e., the O-protonation of amide by H_3O^+, and the second step i.e., the deuteron removal by H_2O, are found to affect the back exchange rate.[3]

Sequence Maps and Peaks Assignment Tables for Equine Myoglobin Using Different Enzymatic Digestion Approach

The identification of the Mb digest fragment is performed using undeuterated Mb, and the detailed experimental procedures for the three digestion approaches could be found in the main manuscript. The sequence coverage of Mb resulted from all the three approaches reached 100%. The sequence maps in this section are created with the aid of the web-based application *MSTools*.[4]

1. Bulk Digestion in Room Conditions

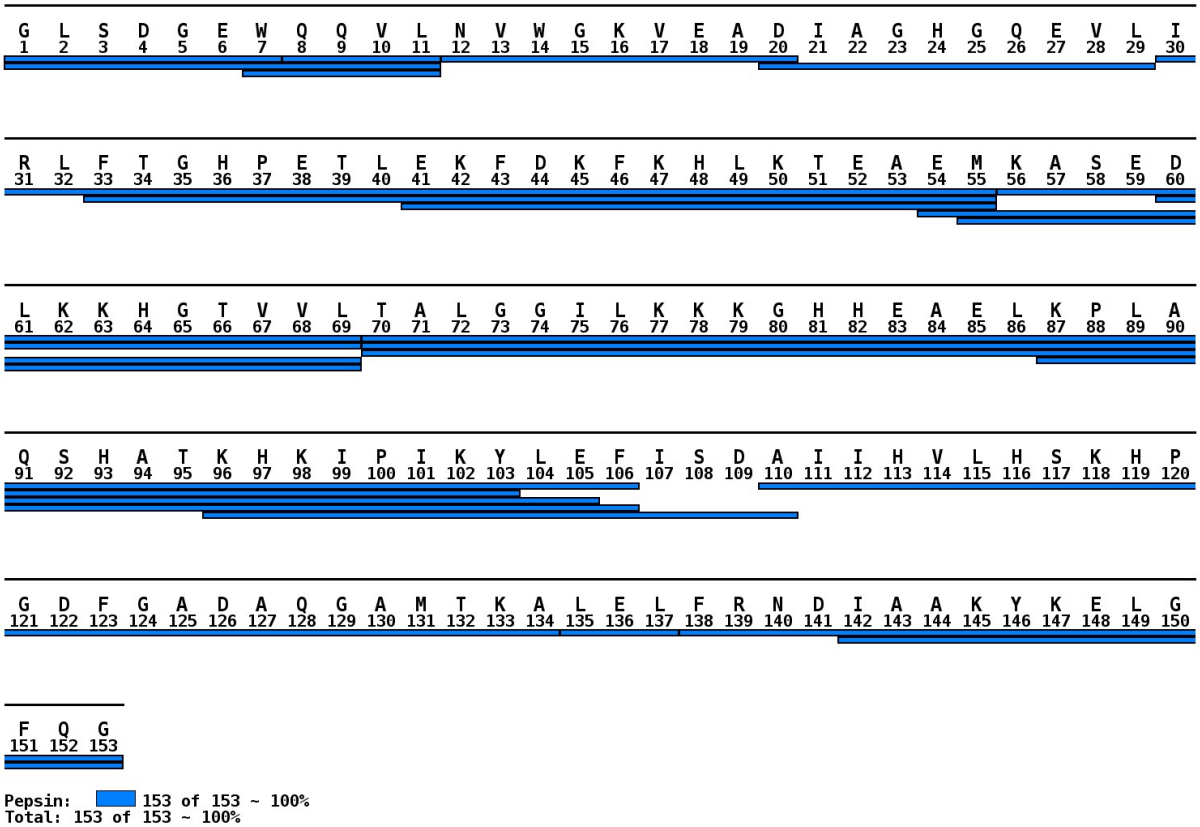

Figure S3. The sequence map resulted from the pepsin digestion of myoglobin conducted in bulk solution for 2 mins under room conditions.

Experimental m/z	Absolute Intensity (a.u.)	Relative Intensity (%)	z	Peak Assignment	Theoretical m/z	Error (ppm)
763.3256	1.16E+08	9.62	1+	1- 7	763.3262	-0.85
1231.5926	7.87E+07	6.55	1+	1- 11	1231.5959	-2.73
673.3681	3.23E+07	2.69	1+	7- 11	673.3673	1.08
487.2876	1.43E+08	11.87	1+	8- 11	487.2880	-0.97
509.2538	3.83E+07	3.19	2+	12- 20	509.2542	-0.80
519.7647	6.45E+07	5.37	2+	20- 29	519.7649	-0.43
1038.5188			1+		1038.5220	-3.15
629.9357	4.08E+08	33.98	5+	30- 55	629.9364	-1.10
787.1673			4+		787.1686	-1.61
1049.2204			3+		1049.2222	-1.65
553.4818	3.53E+08	29.36	5+	33- 55	553.4826	-1.51
691.6004			4+		691.6013	-1.24
921.7973			3+		921.7991	-1.99
627.6564	6.92E+07	5.76	3+	41- 55	627.6579	-2.36
940.9840			2+		940.9829	1.14
595.6569	5.08E+07	4.23	3+	54- 69	595.6579	-1.63
892.9803			2+		892.9829	-2.91
552.6433	4.28E+07	3.56	3+	55- 69	552.6437	-0.73
381.9743	1.02E+09	84.49	4+	56- 69	381.9746	-0.79
508.9631			3+		508.9635	-0.80
762.9403			2+		762.9414	-1.47
370.5609	4.10E+07	3.41	3+	60- 69	370.5613	-1.00
555.3377			2+		555.3380	-0.57
591.1995	9.15E+08	76.10	7+	70-106	591.2003	-1.30
689.5651			6+		689.5657	-0.95
827.2761			5+		827.2773	-1.49
1033.8424			4+		1033.8447	-2.19
1378.1173			3+		1378.1236	-4.59
535.6007	3.31E+08	27.56	7+	70-103	535.6010	-0.52
624.6993			6+		624.6999	-0.87
749.4374			5+		749.4383	-1.22
936.5439			4+		936.5459	-2.12
1248.3904			3+		1248.3919	-1.19
797.8620	1.23E+08	10.20	5+	70-105	797.8636	-2.05
997.0752			4+		997.0776	-2.40
665.0537			6+		665.0543	-0.99
588.0917	5.86E+07	4.87	4+	87-106	588.0921	-0.66
783.7855			3+		783.7868	-1.65
601.3475	8.21E+07	6.83	3+	96-110	601.3458	2.85
643.8325	7.04E+08	58.60	4+	110-134	643.8332	-1.10
858.1069			3+		858.1084	-1.69
1286.6548			2+		1286.6586	-2.95
374.2288	3.45E+08	28.70	1+	135-137	374.2291	-0.84

464.9968	1.07E+09	89.11	4+	138-153	464.9974	-1.25
619.6598			3+		619.6605	-1.14
928.9849			2+		928.9869	-2.08
442.2494	6.19E+07	5.15	3+	142-153	442.2474	4.61
662.8702			2+		662.8672	4.52

Table S5. Peak assignment table for the mass spectrum obtained from the bulk digestion of Mb under room conditions.

2. Water-in-oil Droplet Digestion in Room Conditions

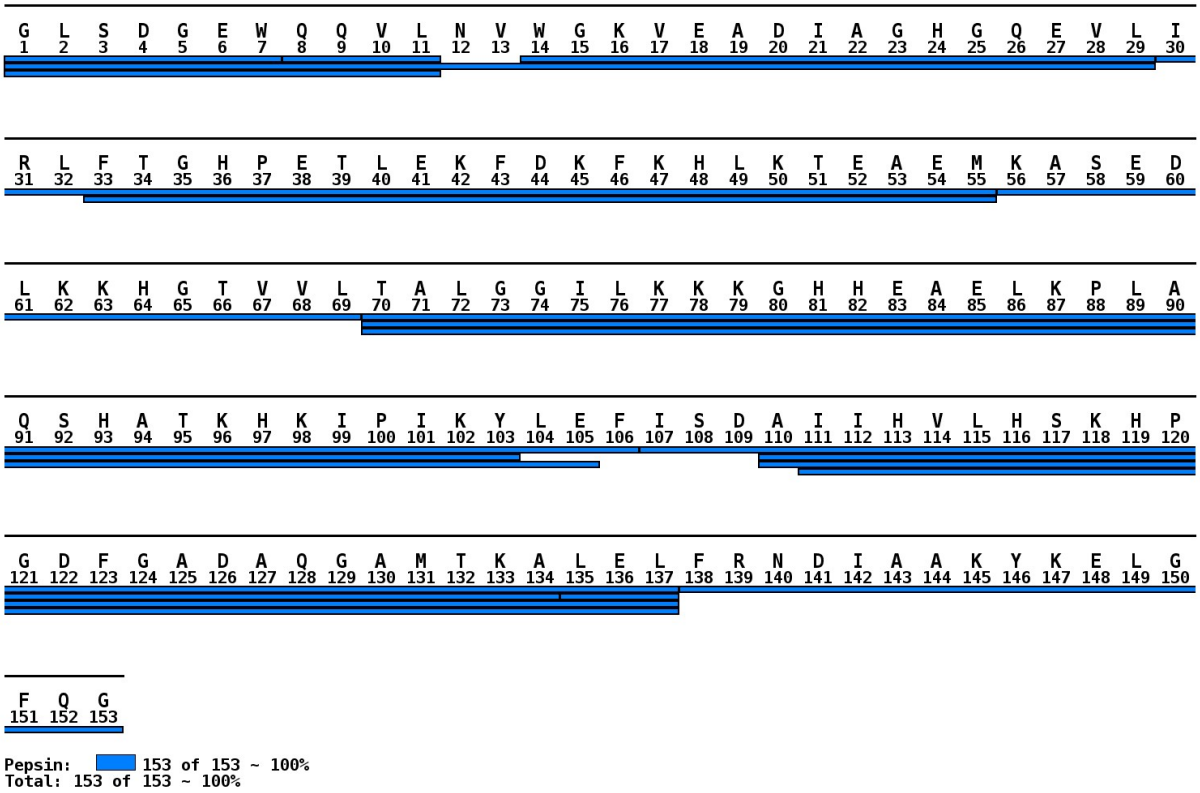

Figure S4. The sequence map resulted from the pepsin digestion of myoglobin conducted in water-in-oil droplet for 2 mins under room conditions.

Experimental m/z	Absolute Intensity (a.u.)	Relative Intensity (%)	z	Peak Assignment	Theoretical m/z	Error (ppm)
763.3261	1.34E+08	4.93	1+	1- 7	763.3262	-0.19
1231.5937	4.18E+08	15.40	1+	1- 11	1231.5959	-1.85
1045.5223	1.38E+08	5.09	3+	1- 29	1045.5235	-1.15
1567.7850			2+		1567.7813	2.38
487.2879	1.17E+08	4.30	1+	8- 11	487.2880	-0.28
570.2936	2.73E+08	10.07	3+	14- 29	570.2938	-0.43
854.9359			2+		854.9368	-1.09
629.9365	5.80E+08	21.35	5+	30- 55	629.9364	0.12
787.1681			4+		787.1686	-0.57
1049.2212			3+		1049.2222	-0.90
1573.3335			2+		1573.3293	2.65
691.6007	1.82E+08	6.70	4+	33- 55	691.6013	-0.87
921.7977			3+		921.7991	-1.49
1382.1943			2+		1382.1947	-0.33
508.9636	9.91E+08	36.50	3+	56- 69	508.9635	0.15
762.9410			2+		762.9414	-0.45
624.6998	1.65E+08	6.09	6+	70-103	624.6999	-0.10
749.4372			5+		749.4383	-1.46
936.5447			4+		936.5459	-1.31
1248.3919			3+		1248.3919	-0.05
797.8630	1.73E+08	6.36	5+	70-105	797.8636	-0.75
997.0765			4+		997.0776	-1.04
1329.1021			3+		1329.1008	0.99
591.2000	2.66E+09	98.08	7+	70-106	591.2003	-0.50
689.5658			6+		689.5657	0.13
827.2769			5+		827.2773	-0.49
1033.8439			4+		1033.8447	-0.77
1378.1223			3+		1378.1236	-0.92
811.4213	5.17E+08	19.03	4+	107-137	811.4217	-0.52
1081.5581			3+		1081.5596	-1.45
643.8331	1.01E+09	37.30	4+	110-134	643.8332	-0.13
858.1077			3+		858.1084	-0.81
1286.6575			2+		1286.6586	-0.88
732.6359	5.57E+08	20.53	4+	110-137	732.6359	0.00
976.5109			3+		976.5119	-1.03
1464.2697			2+		1464.2640	3.89
714.8771	7.87E+07	2.90	4+	111-137	714.8766	0.65
952.8325			3+		952.8329	-0.38
374.2290	5.57E+08	20.50	1+	135-137	374.2291	-0.25
464.9968	2.71E+09	100.00	4+	138-153	464.9974	-1.25
619.6605			3+		619.6605	-0.12
928.9855			2+		928.9869	-1.53
442.2495	3.86E+08	14.22	3+	142-153	442.2474	4.65

Table S6. Peak assignment table for the mass spectrum obtained from the water-in-oil droplet digestion of Mb under room conditions.

3. Bulk Digestion in Ice-water Bath

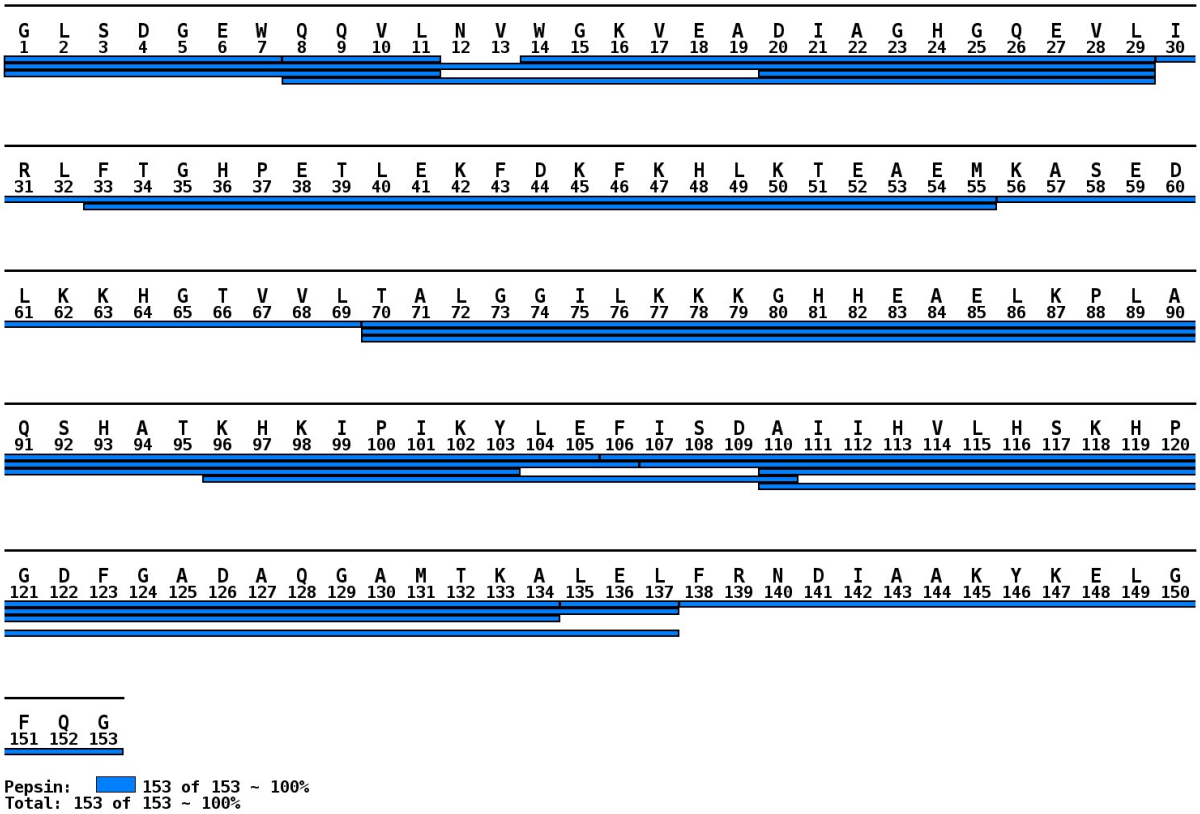

Figure S5. The sequence map resulted from the pepsin digestion of myoglobin conducted in bulk solution for 2 mins in the presence of ice-water bath.

Experimental m/z	Absolute Intensity (a.u.)	Relative Intensity (%)	z	Assignment	Theoretical m/z	Error (ppm)
763.3277	4.27E+07	4.24	1+	1- 7	763.3262	1.93
1231.5958	1.14E+08	11.31	1+	1- 11	1231.5959	-0.14
1045.5263	2.26E+07	2.24	3+	1- 29	1045.5235	2.73
487.2882	6.96E+07	6.90	1+	8- 11	487.2880	0.44
797.4217	4.68E+07	4.64	3+	8- 29	797.4208	1.07
570.2943	1.40E+08	13.93	3+	14- 29	570.2938	0.78
854.9372			2+		854.9368	0.44
519.7654	3.61E+07	3.57	2+	20- 29	519.7649	0.93
629.9368	2.94E+08	29.18	5+	30- 55	629.9364	0.64
787.1695			4+		787.1686	1.15
1049.2231			3+		1049.2222	0.93
691.6021	1.08E+08	10.68	4+	33- 55	691.6013	1.25
921.7995			3+		921.7991	0.43
381.9749	4.38E+08	43.38	4+	56- 69	381.9746	0.68
508.9641			3+		508.9635	1.11
762.9422			2+		762.9414	1.03
591.2004	9.00E+08	89.24	7+	70-106	591.2003	0.09
689.5666			6+		689.5657	1.34
827.2784			5+		827.2773	1.37
1033.8454			4+		1033.8447	0.73
1378.1250			3+		1378.1236	0.99
624.7006	8.01E+07	7.94	6+	70-103	624.6999	1.08
749.4397			5+		749.4383	1.91
936.5459			4+		936.5459	0.04
665.0547	7.77E+07	7.70	6+	70-105	665.0543	0.54
797.8638			5+		797.8636	0.22
601.3485	3.52E+07	3.49	3+	96-110	601.3458	4.58
759.3871	4.28E+07	4.24	4+	106-134	759.3861	1.26
1012.1790			3+		1012.1789	0.18
811.4225	1.96E+08	19.48	4+	107-137	811.4217	1.05
1081.5608			3+		1081.5596	1.04
643.8339	3.95E+08	39.14	4+	110-134	643.8332	1.09
858.1096			3+		858.1084	1.39
732.6374	1.64E+08	16.25	4+	110-137	732.6359	2.09
976.5132			3+		976.5119	1.35
374.2293	2.75E+08	27.29	1+	135-137	374.2291	0.41
464.9974	7.54E+08	74.73	4+	138-153	464.9974	0.04
619.6613			3+		619.6605	1.17
928.9873			2+		928.9869	0.49

Table S7. Peak assignment table for the mass spectrum obtained from the bulk digestion of Mb in the presence of ice-water bath.

Sequence Maps and Peaks Assignment Tables for Bovine Serum Albumin Using Different Enzymatic Digestion Approach

The pepsin digestion of bovine serum albumin (BSA) was conducted in bulk and droplet respectively at room conditions using the same procedures as Mb. The detailed digestion procedures could be found in the main manuscript. The sequence maps in this section are created with the aid of the web-based application *MSTools*.[4]

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1. Bulk Digestion in Room Conditions

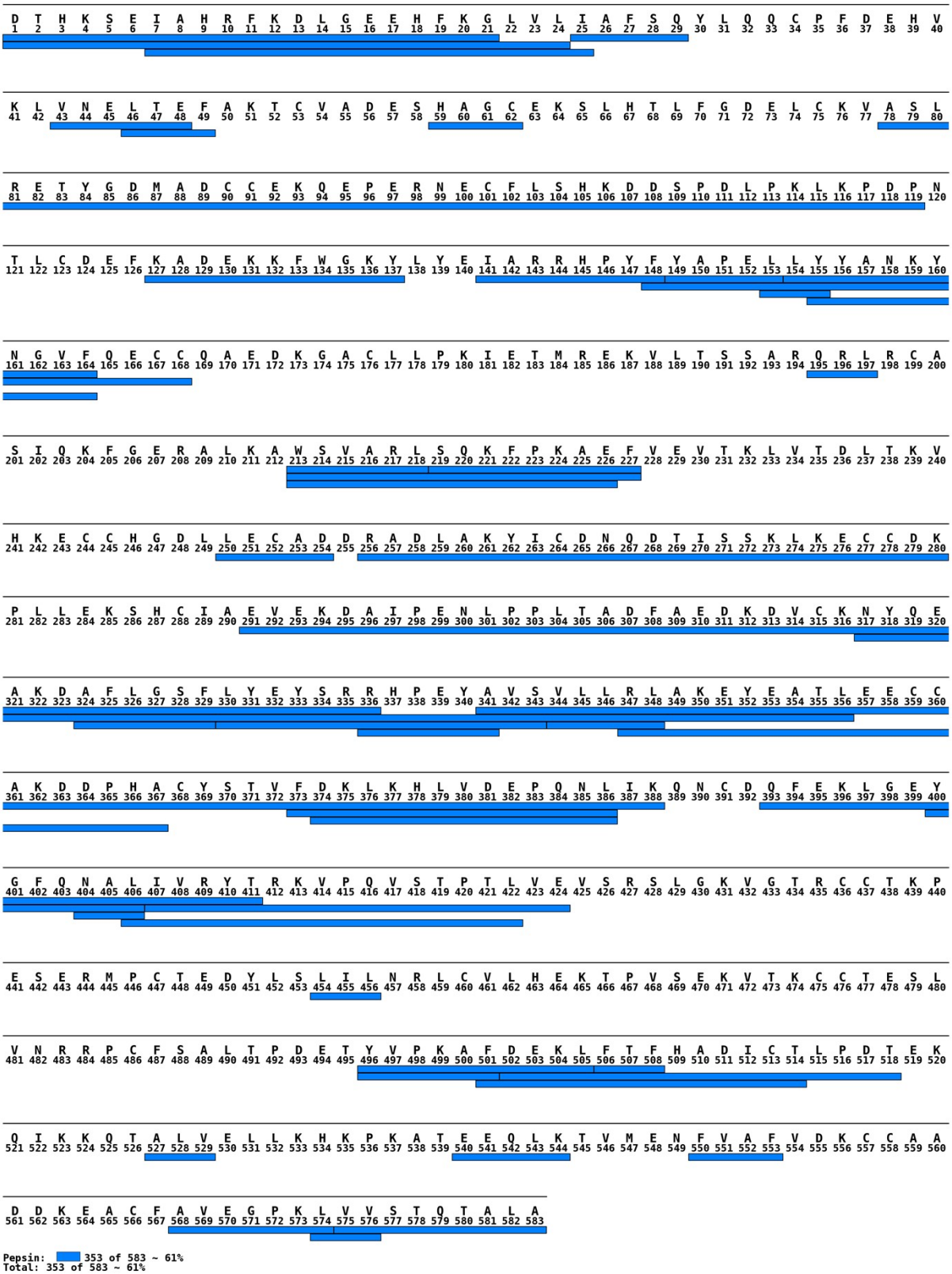

Figure S6. The sequence map resulted from the pepsin digestion of BSA conducted in bulk solution for 2 mins under room conditions.

Experimental m/z	Absolute Intensity (a.u.)	Relative Intensity (%)	z	Assignment	Theoretical m/z	Error (ppm)
497.0515	6.00E+08	56.33	5+	1- 21	497.0518	-0.51
621.0621			4+		621.0628	-1.03
827.7464			3+		827.7477	-1.65
562.0991	2.72E+08	25.49	5+	1- 24	562.0991	0.03
702.3702			4+		702.3719	-2.46
936.1565			3+		936.1599	-3.61
1111.6250	2.16E+08	20.31	2+	7- 25	1111.6264	-1.32
565.2983	7.50E+07	7.04	1+	25- 29	565.2986	-0.66
704.3462	4.52E+07	4.24	1+	43- 48	704.3466	-0.65
509.2610	3.00E+07	2.82	1+	46- 49	509.2611	-0.19
387.1468	7.42E+07	6.96	1+	59- 62	387.1450	4.51
1202.5544	3.00E+07	2.81	4+	78-119	1202.5496	4.05
350.6902	3.76E+08	35.31	4+	127-137	350.6902	-0.13
467.2509			3+		467.2510	-0.33
700.3717			2+		700.3726	-1.26
353.8667	2.23E+08	20.92	3+	141-148	353.8670	-0.67
530.2962			2+		530.2965	-0.54
634.5423	9.01E+07	8.46	4+	148-168	634.5400	3.58
845.7194			3+		845.7174	2.41
592.2971	5.79E+07	5.43	1+	149-153	592.2982	-1.97
408.2497	3.69E+07	3.46	1+	153-155	408.2498	-0.33
676.3376	2.19E+08	20.5	2+	154-164	676.3382	-0.92
619.7957	1.61E+08	15.13	2+	155-164	619.7962	-0.86
416.2619	5.07E+07	4.75	1+	195-197	416.2621	-0.44
366.2139	4.34E+08	40.69	2+	213-218	366.2141	-0.64
731.4197			1+		731.4204	-0.95
598.6616	2.51E+08	23.51	3+	213-227	598.6620	-0.68
897.4868			2+		897.4890	-2.48
549.6385	3.54E+07	3.32	3+	213-226	549.6392	-1.25
361.1944	4.21E+08	39.51	3+	219-227	361.1946	-0.68
541.2876			2+		541.2880	-0.80
550.2179	4.81E+07	4.51	1+	250-254	550.2183	-0.77
954.1298	5.27E+07	4.95	3+	256-280	954.1267	3.24
1065.7213	2.84E+07	2.67	5+	291-336	1065.7210	0.21
1331.9013			4+		1331.8993	1.51
790.9102	2.72E+07	2.56	6+	317-356	790.9082	2.55
590.6249	9.78E+07	9.17	3+	330-343	590.6256	-1.15
885.4327			2+		885.4345	-1.99
641.3294	5.44E+07	5.11	1+	324-329	641.3299	-0.79
772.3734	3.56E+07	3.34	1+	336-341	772.3742	-1.03
908.4698	3.03E+07	2.84	6+	341-388	908.4655	4.71
307.2237	2.42E+08	22.69	2+	344-348	307.2240	-0.90
613.4394			1+		613.4401	-1.13

798.0392	9.44E+07	8.86	3+	347-367	798.0357	4.38
1196.5445			2+		1196.5496	-4.28
565.9739	9.71E+07	9.12	3+	373-386	565.9742	-0.56
848.4553			2+		848.4574	-2.53
516.9510	3.71E+08	34.85	3+	374-386	516.9514	-0.76
774.9222			2+		774.9232	-1.28
569.8004	4.65E+07	4.36	4+	393-411	569.7988	2.81
759.3961			3+		759.3957	0.51
812.3933	1.23E+08	11.57	1+	400-406	812.3943	-1.30
317.1822	2.80E+07	2.63	1+	404-406	317.1825	-0.99
493.5496	1.49E+08	13.99	4+	406-422	493.5498	-0.28
657.7296			3+		657.7304	-1.17
986.0906			2+		986.0917	-1.12
522.3057	5.02E+07	4.71	4+	407-424	522.3065	-1.56
696.0720			3+		696.0727	-0.96
358.2704	9.08E+07	8.52	1+	454-456	358.2706	-0.52
605.3295	8.33E+07	7.81	2+	496-505	605.3299	-0.67
724.4027	4.81E+07	4.51	1+	496-501	724.4034	-1.04
562.9330	6.77E+07	6.35	3+	501-514	562.9331	-0.17
843.8949			2+		843.8958	-1.04
492.2371	5.26E+07	4.93	4+	502-518	492.2376	-1.01
655.9814			3+		655.9808	0.80
414.2029	3.76E+07	3.53	1+	506-508	414.2029	-0.11
302.2078	5.34E+07	5.01	1+	527-529	302.2080	-0.84
646.3405	8.72E+07	8.19	1+	540-544	646.3412	-1.08
483.2606	9.23E+07	8.66	1+	550-553	483.2607	-0.22
357.2137	2.85E+08	26.78	2+	568-574	357.2138	-0.29
713.4193			1+		713.4197	-0.65
330.2391	8.31E+07	7.8	1+	574-576	330.2393	-0.59
889.4977	3.82E+08	35.89	1+	575-583	889.4994	-1.96

Table S8. Peak assignment table for the mass spectrum obtained from the bulk digestion of BSA under room conditions.

2. Water-in-oil Droplet Digestion in Room Conditions

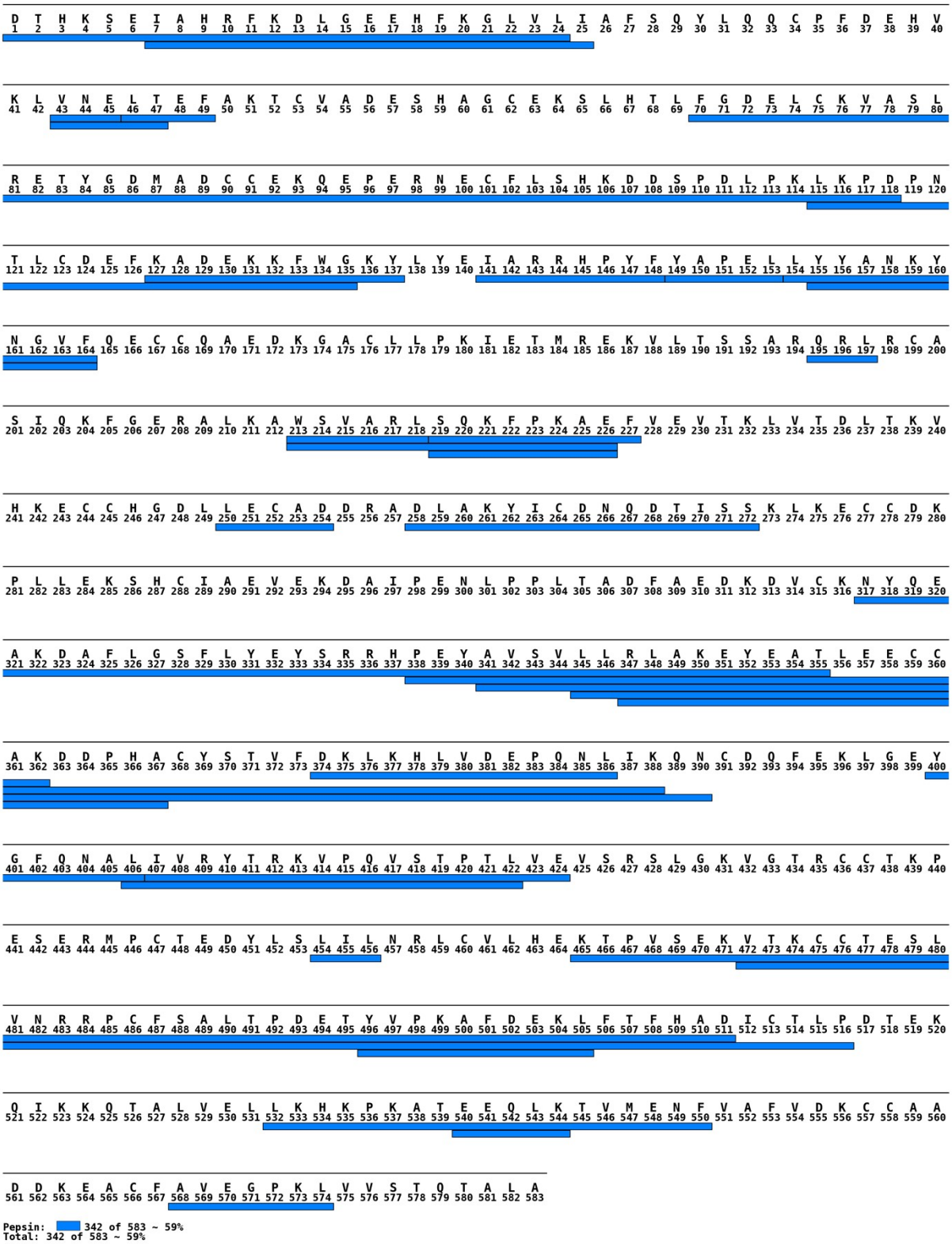

Figure S7. The sequence map resulted from the pepsin digestion of BSA conducted in water-in-oil droplet for 2 mins under room conditions.

Experimental m/z	Absolute Intensity (a.u.)	Relative Intensity (%)	z	Assignment	Theoretical m/z	Error (ppm)
562.0980	3.71E+08	100.00	5+	1- 24	562.0991	-1.86
702.3691			4+		702.3719	-3.95
1111.6227	5.45E+07	14.69	2+	7- 25	1111.6264	-3.35
361.1714	7.71E+06	2.08	1+	43- 45	361.1723	-2.59
575.3032	7.50E+06	2.02	1+	43- 47	575.3040	-1.36
509.2598	1.15E+07	3.11	1+	46- 49	509.2611	-2.62
1121.1152	1.26E+07	3.40	5+	70-118	1121.1139	1.14
989.5120	2.30E+07	6.21	6+	113-160	989.5072	4.80
1187.2042			5+		1187.2071	-2.43
1483.7612			4+		1483.7569	2.91
827.7432	4.26E+07	11.47	3+	115-135	827.7427	0.59
700.3707	3.08E+07	8.31	2+	127-137	700.3726	-2.69
530.2952	3.37E+07	9.07	2+	141-148	530.2965	-2.52
592.2965	9.39E+06	2.53	1+	149-153	592.2982	-3.00
676.3361	3.75E+07	10.10	2+	154-164	676.3382	-3.16
619.7943	2.85E+07	7.67	2+	155-164	619.7962	-3.11
416.2612	1.12E+07	3.02	1+	195-197	416.2621	-2.15
598.6602	2.34E+08	63.02	3+	213-227	598.6620	-2.97
366.2135	1.26E+08	33.97	2+	213-218	366.2141	-1.76
731.4177			1+		731.4204	-3.67
549.6374	1.70E+07	4.58	3+	213-226	549.6392	-3.14
541.2866	1.22E+08	32.96	2+	219-227	541.2880	-2.60
467.7522	7.87E+06	2.12	2+	219-226	467.7538	-3.39
550.2168	1.71E+07	4.61	1+	250-254	550.2183	-2.83
843.3924	6.52E+06	1.76	2+	258-272	843.3885	4.57
926.2702	2.09E+07	5.64	5+	317-355	926.2715	-1.41
943.4827	6.20E+06	1.67	3+	338-362	943.4790	3.88
1089.9594	3.47E+07	9.35	5+	341-388	1089.9570	2.21
1362.1915			4+		1362.1943	-2.07
1333.6719	1.96E+07	5.29	4+	345-390	1333.6682	2.82
798.0367	7.44E+06	2.01	3+	347-367	798.0357	1.32
516.9499	4.09E+07	11.02	3+	374-386	516.9514	-3.05
774.9205			2+		774.9232	-3.50
812.3904	3.64E+07	9.81	1+	400-406	812.3943	-4.86
657.7284	5.81E+07	15.65	3+	406-422	657.7304	-2.97
986.0876			2+		986.0917	-4.10
696.0708	2.31E+07	6.23	3+	407-424	696.0727	-2.67
1043.6013			2+		1043.6051	-3.68
885.4284	8.14E+06	2.19	2+	442-456	885.4266	1.96
358.2701	1.64E+07	4.43	1+	454-456	358.2706	-1.55
1070.5365	1.11E+07	2.98	5+	465-511	1070.5349	1.44
1337.9153			4+		1337.9167	-1.04
1022.1059	1.11E+07	2.99	5+	472-516	1022.1038	2.00

605.3279	3.00E+07	8.08	2+	496-505	605.3299	-3.33
873.4741	1.31E+08	35.29	2+	512-526	873.4775	-3.86
757.7434	3.86E+06	1.04	3+	532-550	757.7443	-1.17
646.3388	2.84E+07	7.67	1+	540-544	646.3412	-3.72
713.4174	1.39E+07	3.74	1+	568-574	713.4197	-3.20

Table S9. Peak assignment table for the mass spectrum obtained from the water-in-oil droplet digestion of BSA under room conditions.

Evaluation on the Effect of Droplet Size on the Deuterium Uptake

In this section, the experimental procedures illustrated in Figure 2B of the main manuscript is adopted to evaluate the effect of droplet size on the deuterium uptake. Experimentally, a 20 μM fully deuterated Mb sample was first prepared by incubating 500 μM Mb stock solution (prepared in 1.25M ammonium acetate) in 96% (v/v) D_2O at 65°C for 3 hours. Pepsin digestion of the fully deuterated Mb was then conducted by adding 2.5 μL of the fully deuterated Mb into 22.5 μL 11.1μM pepsin solution prepared in 0.5% formic acid (pH~2.0). The volume and molar protein-to-pepsin ratios were 1-to-9 and 1-to-5, respectively. The pepsin digestion of Mb was carried out in a capped Eppendorf tube for 2 mins, followed by the addition of 25 μL acetonitrile (ACN) for digestion quenching. 1.0 μL or 0.5 μL of this deuterated Mb digest was then added into the cyclohexane reservoir preloaded in a cut Eppendorf tube. The back exchange reaction of the deuterated Mb digest was allowed to proceed in the water-in-oil-droplet for a predefined duration, followed by the LMJ-SSP-MS analysis. The experiment was performed using four time points, i.e., 0, 2, 5 and 10 mins.

Figure S7 showed the deuterium uptake recorded the deuterium uptake when 1.0 μL (red bars) and 0.5 μL (blue bars) of deuterated Mb digest was added into the cyclohexane reservoir. The two droplet volumes resulted in highly comparable deuterium uptake for all the fragments across time.

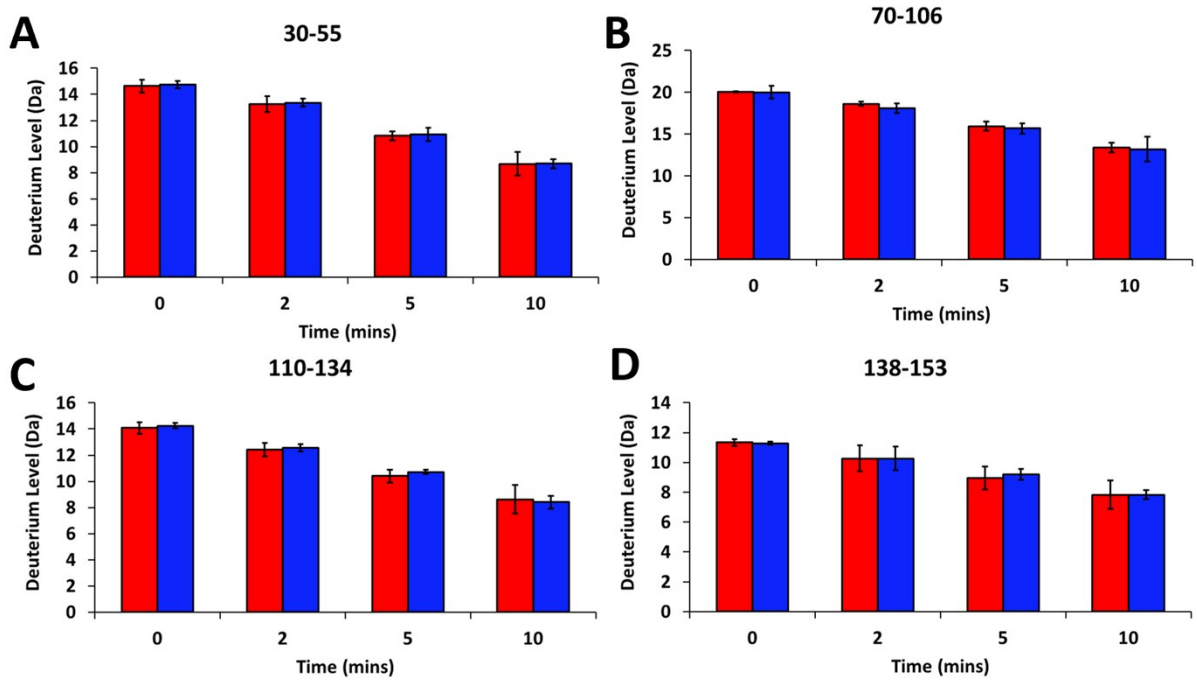

Figure S8. Bar charts for the back exchange reaction of deuterated Mb digest fragments. Bar charts for the back exchange reaction of deuterated Mb digest fragment (A) 30-55; (B) 70-106; (C) 110-134 and (D) 138-153. The red and blue bars represent the deuterium uptake when 1.0 μL and 0.5 μL of deuterated Mb digest was added into the cyclohexane reservoir, respectively. Each data point reports the average of three trials, and the error bars represent the standard deviation of three trials.

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