

## Effect of centrifugation on tryptic protein digestion

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**Abstract:** This study investigated the effect of centrifugation on tryptic digestion. This was done by applying different centrifugation speeds (6,000, 8,000, 10,000, 20,000, and 30,000×g) over various durations (0, 10, 20, 30, 40, 50, and 60 min) to digest two model proteins - cytochrome *c* and myoglobin. The intact proteins and resulting peptides were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Centrifugation greatly improved the tryptic digestion efficiency of cytochrome *c*, where either an increase in centrifugation speed or in digestion duration significantly improved the digestion of cytochrome *c*. However, centrifugation did not noticeably improve the digestion of myoglobin; 16 h of centrifuge-assisted tryptic digestion at 30,000×g barely removed the myoglobin protein peak. Similar results were also obtained when using conventional tryptic digestion with gentle mixing. When acetonitrile (ACN) was added to make 10% ACN buffer solutions, the myoglobin protein peak disappeared after 6 h of digestion using both centrifuge-assisted and conventional tryptic digestions.

**Key words:** centrifuge, centrifugation, trypsin, digestion, proteins, MALDI

### 1. Introduction

Trypsin is currently the enzyme most commonly used to cleave proteins into peptides. Tryptic protein digestion, followed by identification using mass spectrometry, is an essential step in many proteomic studies. Generating peptides from proteins is very important in proteomics, as mass spectrometers cannot comprehensively characterize whole proteins due to their limited sensitivity and accuracy. Protein digestion using trypsin is commonly performed overnight at

37 °C. To expedite this tryptic digestion process, previous studies have used methods such as microwave irradiation,<sup>1,2</sup> the application of ultrasound,<sup>3</sup> vortexing,<sup>4</sup> and pressure.<sup>5,6</sup>

Here, we studied the effect of centrifugation on improving the tryptic digestion of cytochrome *c* and myoglobin. Centrifuge-assisted tryptic digestion was conducted at 37 °C under various centrifugation speeds (10,000×g, 20,000×g, and 30,000×g) and digestion times (1, 2, 3, 6, and 16 h). The effect of acetonitrile (ACN) on the centrifuge-assisted tryptic

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digestion of myoglobin was also evaluated.

## 2. Experimental

### 2.1. Materials

Chemicals, including formic acid (96 %), 2,5-dihydroxybenzoic acid (2,5-DHB), phosphoric acid (85 %), cytochrome *c* from equine heart (mw ~12.5 kDa), myoglobin from horse skeletal muscle (mw ~17.0 kDa), ACN and ammonium bicarbonate ( $\geq 99.0\%$ ), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was obtained from Promega (Madison, WI, USA).

### 2.2. Preparation of protein samples

Stock solutions containing 10 mg/mL of equine heart cytochrome *c* in 50 mM ammonium bicarbonate, and 10 mg/mL of horse skeletal muscle myoglobin in 50 mM ammonium bicarbonate, were first prepared. A sample solution (1 mg/mL) was prepared by combining 100  $\mu$ L of stock solution with 900  $\mu$ L of the 50 mM ammonium bicarbonate buffer solution.

### 2.3. Centrifuge-assisted tryptic digestion and conventional tryptic digestion of cytochrome *c*

The centrifuge-assisted tryptic digestion of cytochrome *c* was conducted at 37 °C by centrifuging at 10,000 $\times$ g over six different digestion times (10, 20, 30, 40, 50, or 60 min). This was done using the sample solution (96  $\mu$ L of 1 mg/mL) with 4  $\mu$ L trypsin (0.5  $\mu$ g/ $\mu$ L). Prior to centrifuge-assisted tryptic digestion, trypsin sample solutions were briefly vortexed and centrifuged at 2,000 $\times$ g for 3 s. Centrifuge-assisted tryptic digestion was halted by adding 1  $\mu$ L of formic acid. To find the optimum centrifugation speed, various centrifuge speeds (including 6,000 $\times$ g, 8,000 $\times$ g, or 10,000 $\times$ g) were used for 1 h at 37 °C.

The conventional tryptic digestion of cytochrome *c* was performed in a manner similar to centrifuge-assisted tryptic digestion, except that gentle mixing, consisting of 350 rpm applied by a mixer (Thermomixer compact from Eppendorf) was applied (instead of centrifugation) over digestion times of 1, 2, 3, 6, and 16 h.

### 2.4. Effect of ACN on centrifuge-assisted tryptic digestion of myoglobin

To study the effect of ACN, a 10 % ACN digestion buffer solution was prepared by adding ACN to the digestion buffer solution. The centrifuge-assisted tryptic digestion of myoglobin was conducted at 37 °C for 6 h using a centrifugation speed of 30,000 $\times$ g. Prior to centrifuge-assisted tryptic digestion, samples with trypsin were briefly vortexed, followed by centrifugation as described previously. Reactions were halted by adding 1  $\mu$ L formic acid.

### 2.5. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis

Mass spectra were obtained using an Applied Biosystems MALDI-TOF mass spectrometer (Voyager DE-STR) with a 337 nm nitrogen laser in linear (or reflectron) positive ion mode. A 2,5-DHB was used as a MALDI matrix. This 2,5-DHB matrix was prepared by dissolving 10 mg of 2,5-DHB in 500  $\mu$ L of ACN, 10  $\mu$ L of phosphoric acid, and 490  $\mu$ L of water.<sup>7</sup> To prepare sample spots, 1.5  $\mu$ L mixture of sample and matrix (1:1) was loaded onto the MALDI plate, and then dried in a vacuum chamber (Module 3180C, Hanil). To check for the presence of proteins under various digestion times and centrifugation speeds, mass spectra were obtained at a *m/z* spanning 3,000 to 25,000 in linear positive ion mode. To confirm the presence of peptide peaks, mass spectra were obtained over a *m/z* from 500 to 3,000 in reflectron positive ion mode.

Peptide peaks within the mass spectra were identified by comparing the masses of observed peptide peaks with theoretical peptide peaks; these theoretical peak masses were acquired from PeptideMass ([http://web.expasy.org/peptide\\_mass/](http://web.expasy.org/peptide_mass/)) using CYC\_horse (p00004) and MYG\_horse (p68082) for the respective cytochrome *c* and myoglobin entry names (accession numbers).

## 3. Results and Discussion

### 3.1. Effect of centrifugation speed on centrifuge-assisted tryptic digestion of cytochrome *c*

The effect of centrifugation speed on the centrifuge-

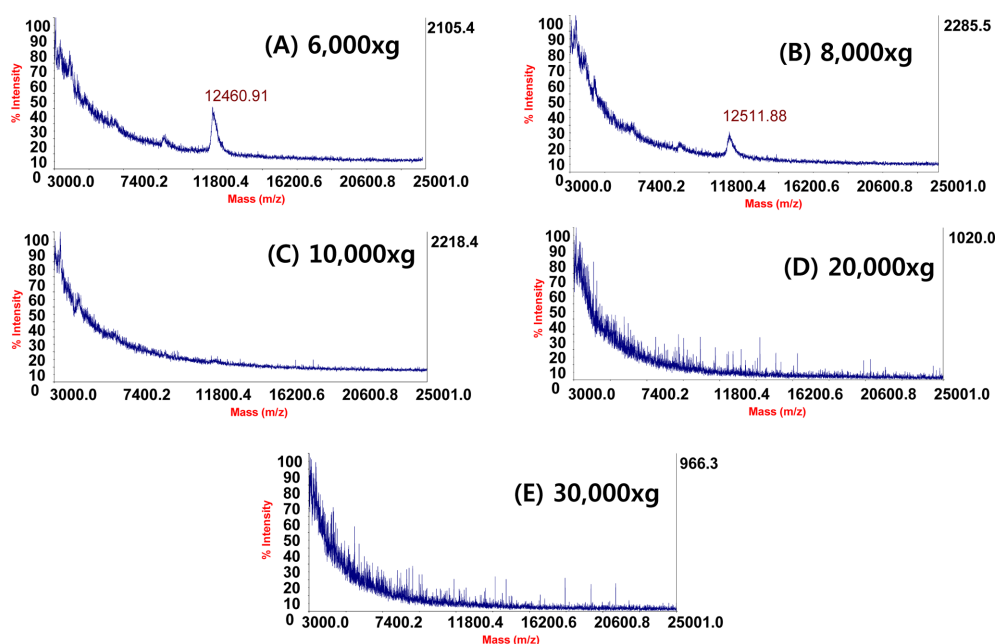


Fig. 1. MALDI mass spectra of cytochrome *c* after 1 h of centrifuge-assisted tryptic digestion at centrifugation speeds of (A) 6,000 $\times$ g, (B) 8,000 $\times$ g, (C) 10,000 $\times$ g, (D) 20,000 $\times$ g and (E) 30,000 $\times$ g (temperature=37 °C).

assisted tryptic digestion of cytochrome *c* was investigated by using different centrifugation speeds (6,000, 8,000, 10,000, 20,000, and 30,000 $\times$ g) during tryptic digestion. Fig. 1 shows the MALDI mass spectra of cytochrome *c* prepared using 1 h of centrifuge-assisted tryptic digestion at 37 °C over five different centrifugation speeds (6,000, 8,000, 10,000, 20,000 and 30,000 $\times$ g). This spectra shows that increasing the centrifugation speed also increases the centrifuge-assisted tryptic digestion efficiency. Fig. 1 also shows that the 1-h centrifuge-assisted tryptic digestion of cytochrome *c* requires a centrifuge speed of at least 10,000 $\times$ g for complete digestion.

Fig. 2 shows MALDI mass spectra of peptide peaks from 1 h of centrifuge-assisted tryptic digestion of cytochrome *c* at 37 °C with a centrifugation speed of 10,000 $\times$ g. Table 1 summarizes peptides identified after 1 h of centrifuge-assisted tryptic digestion of cytochrome *c* at 37 °C using several centrifugation speeds (6,000 $\times$ g, 8,000 $\times$ g, 10,000 $\times$ g, 20,000 $\times$ g, and 30,000 $\times$ g). Three miscleaved peptides (residues 27-40, residues 40-56, and residues 41-56) were observed only within the two lowest centrifuge speeds (6,000

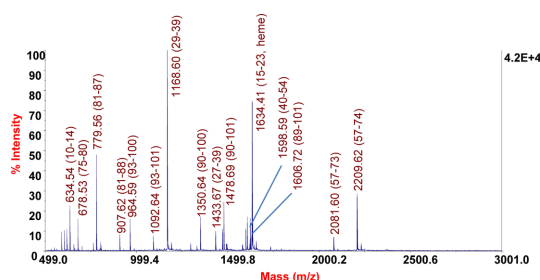


Fig. 2. MALDI mass spectrum of cytochrome *c* produced after centrifuge-assisted tryptic digestion at 37 °C and a speed of 10,000 $\times$ g over 1 h. The *m/z* values of representative peptides are provided alongside sequence information.

and 8,000 $\times$ g); these peptides were not present within high centrifugation speeds (10,000 $\times$ g, 20,000 $\times$ g, and 30,000 $\times$ g), confirming that digestion is more complete at higher centrifugation speeds.

### 3.2. Time dependence of centrifuge-assisted tryptic digestion of cytochrome *c*

To determine the time dependence of centrifuge-assisted tryptic digestion, different digestion times were used at a centrifuge speed of 10,000 $\times$ g. Fig. 3

Table 1. Peptide sequence information obtained after 1 h of centrifuge-assisted tryptic digestion of cytochrome *c* at 37°C using centrifuge speeds of 6,000×g, 8,000×g, 10,000×g, 20,000×g, and 30,000×g

Start	End	Peptide sequence	Number of missed cleavages	Experimental [M+H] <sup>+</sup>	Theoretical monoisotopic [M+H] <sup>+</sup>	Centrifugation speed (×g)				
						6,000	8,000	10,000	20,000	30,000
2	8	GDVEK GK	1	731.63	732.39	-	-	-	O <sup>a)</sup>	O
9	14	KIFVQK	1	762.71	762.49	O	O	O	O	O
10	14	IFVQK	0	634.70	634.39	O	O	O	O	O
15	23	CAQCHTVEK + Heme <sup>b)</sup>	1	1633.08	1633.61	O	O	O	O	O
27	39	HKTGPNLHGLFGR	1	1433.87	1433.78	-	O	O	O	O
27	40	HKTGPNLHGLFGRK	2	1561.43	1561.87	O	O	-	-	-
29	39	TGPNLHGLFGR	0	1168.54	1168.62	O	O	O	O	O
29	40	TGPNLHGLFGRK	1	1296.52	1296.72	O	O	O	O	-
40	54	KTGQAPGFYTDANK	1	1598.31	1598.78	O	O	O	O	O
40	56	KTGQAPGFYTDANKNK	2	1840.17	1840.92	O	O	-	-	-
41	54	TGQAPGFYTDANK	0	1470.31	1470.69	O	O	O	O	O
41	56	TGQAPGFYTDANKNK	1	1712.22	1712.82	O	O	-	-	-
57	61	GITWK	0	604.68	604.35	O	O	O	O	O
57	73	GITWKEETLMEYLENPK	1	2081.00	2081.03	O	O	O	O	O
57	74	GITWKEETLMEYLENPKK	2	2209.39	2209.12	O	O	O	O	O
62	73	EETLMEYLENPK	0	1495.30	1495.70	O	O	O	O	O
62	74	EETLMEYLENPKK	1	1623.27	1623.79	O	O	O	O	O
74	80	KYIPGTK	1	806.66	806.48	O	O	O	O	O
74	92	KYIPGTKMIFAGIKKKTER	5	2209.95	2209.29	O	O	O	O	O
75	80	YIPGTK	0	678.65	678.38	O	O	O	O	O
75	92	YIPGTKMIFAGIKKKTER	4	2080.06	2081.03	O	O	O	O	O
81	87	MIFAGIK	0	779.65	779.45	O	O	O	O	O
81	88	MIFAGIKK	1	907.66	907.54	O	O	O	O	O
88	92	KKTER	2	661.70	661.40	O	O	-	O	O
88	101	KKTEREDLIAYLKK	4	1735.40	1735.01	O	O	O	O	O
89	92	KTER	1	533.69	533.30	O	O	-	O	O
89	101	KTEREDLIAYLKK	3	1606.43	1606.92	O	O	O	O	O
90	100	TEREDLIAYLK	1	1350.47	1350.73	O	O	O	O	O
90	101	TEREDLIAYLKK	2	1478.45	1478.82	O	O	O	O	O
93	100	EDLIAYLK	0	964.60	964.53	O	O	O	O	O
93	101	EDLIAYLKK	1	1092.60	1092.63	O	O	O	O	O
93	105	EDLIAYLKKATNE	2	1506.16	1507.80	-	-	-	O	-
101	105	KATNE	1	562.64	562.28	O	O	O	O	O
Number of identified peptides						30	31	25	30	28

a) "o" means the detection of the corresponding peptide.

b) The peptide contains a heme group.

shows MALDI mass spectra of cytochrome *c* prepared using centrifuge-assisted tryptic digestion (temperature =37 °C; speed=10,000×g) under 0, 10, 20, 30, 40, 50, and 60 min of digestion time. The mass spectrum at 0 min shows cytochrome *c* without trypsin treatment; this cytochrome *c* peak gradually decreased as the digestion duration increased, disappearing completely

after 60 min.

### 3.3. Centrifuge-assisted tryptic digestion of myoglobin

Previous studies have shown that the tryptic digestion of myoglobin is poor,<sup>4</sup> and can be expedited by the use of ACN. To investigate whether centrifuging can

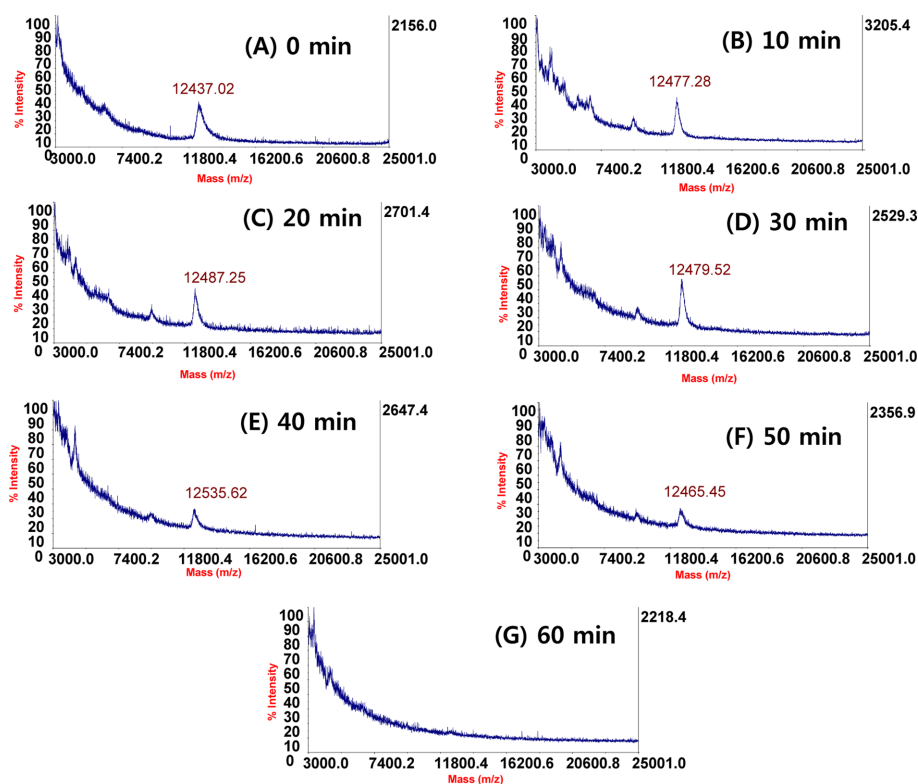


Fig. 3. MALDI mass spectra of cytochrome *c* produced after centrifuge-assisted tryptic digestion at 10,000xg for (A) 0 min, (B) 10 min, (C) 20 min, (D) 30 min, (E) 40 min, (F) 50 min, and (G) 60 min.

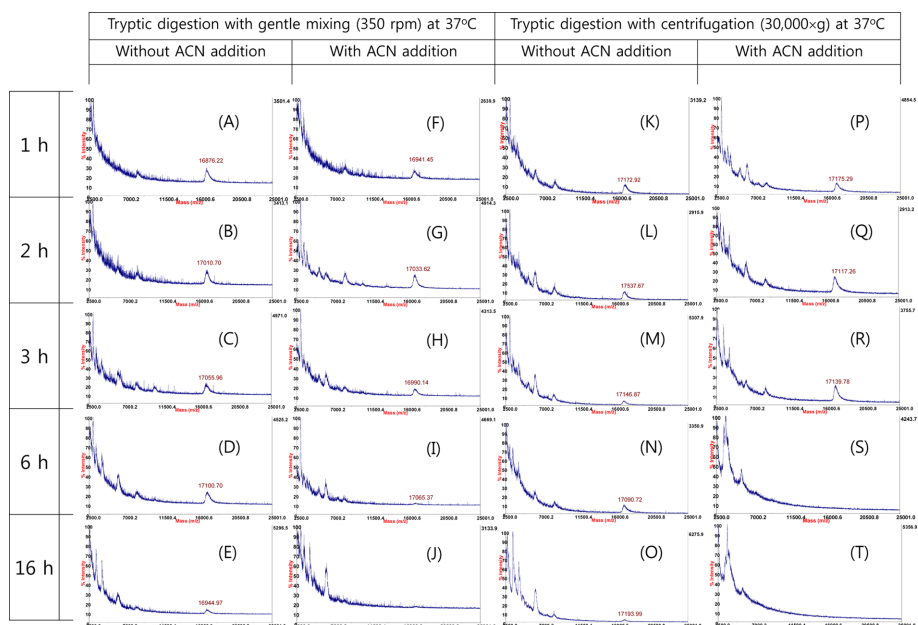


Fig. 4. MALDI mass spectra of myoglobin prepared using: 1) conventional tryptic digestion (A-E) without ACN and (F-J) with ACN; and 2) centrifuge-assisted tryptic digestion at 30,000xg (K-O) without ACN and (P-T) with ACN over 1, 2, 3, 6, and 16 h of digestion.

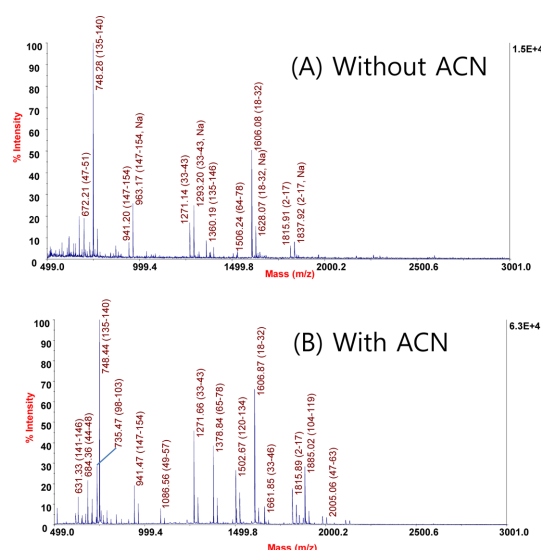


Fig. 5. MALDI mass spectra of myoglobin peptides prepared using centrifuge-assisted tryptic digestion at 30,000 $\times$ g and 37 °C for 6 h in a buffer solution (A) without ACN and (A) with 10 % ACN. The  $m/z$  values of representative peptides are provided alongside sequence information.

increase the tryptic digestion efficiency of myoglobin, we used conventional tryptic digestion (with gentle mixing at 350 rpm) and centrifuge-assisted tryptic digestion (with a centrifuge speed of 30,000 $\times$ g) performed both in the absence and presence of ACN under several digestion times (specifically, 1, 2, 3, 6, and 16 h), as shown in Fig. 4. Increasing the duration of digestion decreased the intensity of the myoglobin peak. However, neither conventional tryptic digestion nor centrifuge-assisted tryptic digestion completely removed the myoglobin protein peak in the absence of ACN. Small additions of ACN (for example, the 10 % ACN buffer solution) significantly expedited the centrifuge-assisted tryptic digestion efficiency of myoglobin, completely removing the myoglobin peak after 6 h. Interestingly, no significant differences in the MALDI mass spectra of myoglobin were observed between conventional tryptic digestion and centrifuge-assisted tryptic digestion. Fig. 5 thus shows the effect of ACN on the centrifuge-assisted tryptic digestion of myoglobin after 6 h (temperature =37 °C;

speed=30,000 $\times$ g), where many more myoglobin peptides were identified after treatment with ACN.

## 4. Conclusions

Centrifuging greatly impacted the tryptic digestion of cytochrome *c*, where digestion efficiency improved as centrifugation speed and centrifuge duration increased. However, centrifugation has little impact on the tryptic digestion of myoglobin, where similar digestion efficiencies were observed between centrifuge-assisted and conventional tryptic digestion. Improved digestion efficiency of cytochrome *c* is believed to be due to denaturation caused by high speed centrifugation, and increased collision frequency between cytochrome *c* and trypsin; conversely, myoglobin is too robust to be denatured at the centrifugation speeds used within this study.

## Acknowledgements

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