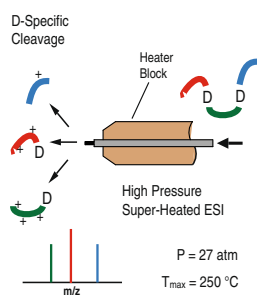


## RESEARCH ARTICLE

# Rapid Online Non-Enzymatic Protein Digestion Analysis with High Pressure Superheated ESI-MS

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**Abstract.** Recently, we reported a new ESI ion source that could electrospray the super-heated aqueous solution with liquid temperature much higher than the normal boiling point (*J. Am. Soc. Mass Spectrom.* **25**, 1862–1869). The boiling of liquid was prevented by pressurizing the ion source to a pressure greater than atmospheric pressure. The maximum operating pressure in our previous prototype was 11atm, and the highest achievable temperature was 180°C. In this paper, a more compact prototype that can operate up to 27atm and 250°C liquid temperatures is constructed, and reproducible MS acquisition can be extended to electrospray temperatures that have never before been tested. Here, we apply this super-heated ESI source to the rapid online protein digestion MS. The sample solution is rapidly heated when flowing

through a heated ESI capillary, and the digestion products are ionized by ESI in situ when the solution emerges from the tip of the heated capillary. With weak acid such as formic acid as solution, the thermally accelerated digestion (acid hydrolysis) has the selective cleavage at the aspartate (Asp, D) residue sites. The residence time of liquid within the active heating region is about 20s. The online operation eliminates the need to transfer the sample from the digestion reactor, and the output of the digestive reaction can be monitored and manipulated by the solution flow rate and heater temperature in a near real-time basis.

**Keywords:** High pressure electrospray, Pressurized superheated water, Site-specific protein Digestion, Non-enzymatic digestion, Acid hydrolysis

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## Introduction

The hydrolysis of protein or peptide using weak acid that selectively cleaves at either or both sides of aspartate residues (Asp, D) is commonly called non-enzymatic digestion or chemical digestion. This effect was known as early as 1950 during the hydrolysis of protein in acetic acid or oxalic acid solution at boiling temperature [1]. Same effect is also found in hot diluted hydrochloric acid, but not in strong acid at room temperature [2]. Some discussions on the mechanism behind this selective cleavage are given in references [2–4]. Although not as popular as the enzymatic method, acidic hydrolysis is gaining increasing attention for bottom-up proteomics, and the existing protein database can also be used to cover this

cleavage pattern. Commonly used reagents in the recent literature are formic acid [5] and acetic acid [6, 7]. Hydrolysis using acidic MALDI matrices is also reported to give some similar effect [8].

Unlike enzyme, which is temperature sensitive and cannot withstand high temperature, acidic hydrolysis can be accelerated by heating the liquid to boiling point or above. Polar solvents like aqueous solution can be efficiently and uniformly heated by microwave excitation at 2.45GHz, and there is rich literature on the microwave-assisted chemical digestion [6, 7, 9–14]. The digestion process that typically requires several hours to complete can be accelerated to about 5–10min by incubating the sample in the microwave oven. ESI-MS of the digestion product can be done after cooling down the sample to near room temperature and mixing it with compatible solvent for electrospray.

Heated electrospray ion source can, in principle, be the online reactor for the chemical digestion. In fact, ESI with heated capillary had been done by several research groups to improve the desolvation efficiency [15, 16], and to study the

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conformation change of proteins under thermal activation [17–19]. However, heating of electrospray ion source under atmospheric pressure is limited to the normal boiling point of the solvent, which is 100°C for water. The boiling takes place when the vapor pressure of the liquid at a given temperature equals the ambient gas pressure. The boiling point can be raised beyond its standard atmospheric pressure value by pressurizing the liquid container. When the pressurized water is heated above 100°C, there is a considerable change in the surface tension [20], conductivity [21], dielectric constants [22, 23], as well as water ionization constant,  $K_w$  [24]. Above 200°C, water can even dissolve hydrophobic compounds like aromatic hydrocarbon as efficiently as organic solvents [25] and had been used in liquid extraction and liquid chromatography [26–28].

A series of high pressure ion sources had been developed in our laboratory to improve the performance of ESI [29, 30], nanoESI [31], and atmospheric pressure chemical ionization (APCI) [32]. It had also been used to implement field desorption (FD), an ESI-like ionization from molten sample without the use of any solvent [33]. For ESI-based ionization, the improvement is primarily due to the increase of dielectric strength under high pressure that prevents the occurrence of gaseous breakdown. Under an optimized pressure, it is also possible to sustain a nanoESI level flow rate of ~10nL/min even with large disposable plastic tips of ~100µm inner diameter as emitter [34].

By raising the ion source pressure above 1atm with nitrogen, we had previously demonstrated the electrospray of aqueous solution with liquid temperature above 100°C. Limited by the maximum operating pressure of 11atm, the highest temperature at which the electrospray could remain stable was around 180°C [35]. To extend the accessible temperature, a second prototype is constructed in this study to operate under 27atm, with a maximum temperature of 250°C. Here we demonstrate

its potential use in the thermally accelerated site-specific chemical digestion.

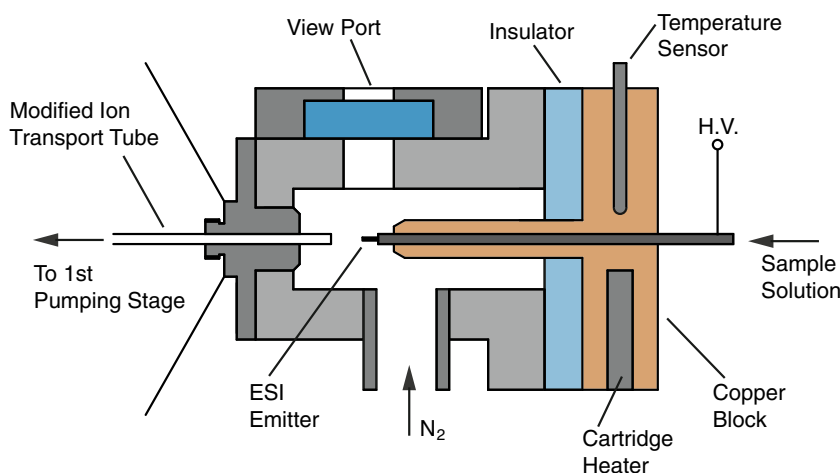
## Experimental

### Mass Spectrometer

The experiment was conducted using a bench top Orbitrap (Exactive, Thermo Fisher Scientific, Bremen, Germany). A Roots booster pump with variable pumping speed (PMB 003C; ULVAC, Kanagawa, Japan) was added to increase the pumping speed for the first pumping stage of the mass spectrometer. The pumping speed of the booster pump was controlled by a variable frequency controller (VF-AS1; Toshiba Schneider Inverter, Nagoya, Japan), and the pressure in the first pumping stage was maintained at ~1.4mbar when the ion source pressure was raised above 1atm. The settings for the Exactive-Orbitrap were as follow: temperature for the ion transport tube was 300°C, the capillary voltage and tube lens voltage were 50V, and skimmer voltage was 20V. The maximum ion injection time was 100ms. The scanning mode for mass spectrum was set for highest mass resolution.

### High Pressure ESI Source

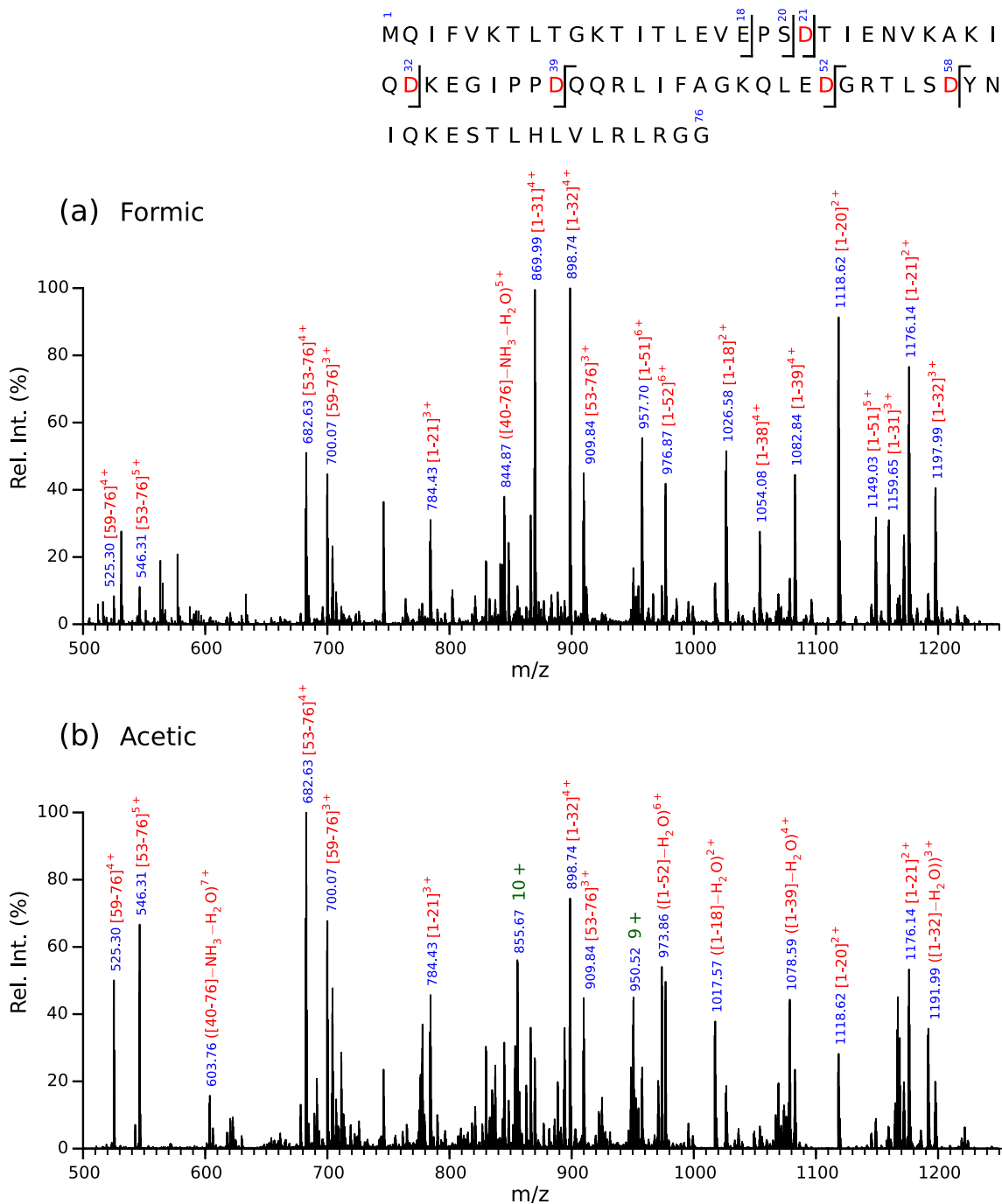
The schematic of the high pressure ESI source is shown in Figure 1. The design is quite similar with our previous prototype, but it is smaller and safer to operate at high pressure and high temperature. Photographs showing the ion source are depicted in Supplementary Figures S1–S3. The material for the main chamber was duralumin (hard aluminum alloy) and the view ports were made of quartz. A stainless steel capillary (i.d. 100µm, o.d. 200µm, and 2cm long from Misumi, Tokyo, Japan) was used as ESI emitter. It was attached to another stainless steel capillary (i.d. 250µm, o.d. 1/16in.; GL Sciences,



**Figure 1.** Simplified schematic of the high pressure superheated ESI. The ion source chamber is pressurized to 27 atm with nitrogen, and the aqueous solution can be maintained at liquid state up to 250°C. The ion source is connected to the mass spectrometer (Orbitrap-Exactive) via a modified ion transport tube with inner diameter of 0.25 mm

Tokyo, Japan) using precision crimping tool. The 1/16in. capillary together with the ESI capillary was inserted to a copper block, and all gaps were filled with thermal grease (silicone and zinc oxide-based). The copper block was heated with cartridge heaters and the temperature was monitored with a platinum temperature sensor. The power supply to the temperature controller (OMRON, Kyoto, Japan) was electrically isolated from the ground using an isolation transformer.

A custom-made ion transport tube with an i.d. of 0.25mm was used to couple the ion source to the Orbitrap. Sample solution was pumped through the ESI capillary using a syringe pump with high linear force (PHD 4400, Harvard Apparatus, Holliston, MA, USA), and the flow rate of sample solution was 4–10 $\mu$ L/min. The length of the heated capillary that was embedded within the copper heater block was 30mm. With capillary i.d. of 0.25mm, the residence time of liquid that stays



**Figure 2.** High pressure ESI mass spectra of bovine ubiquitin in (a) formic acid aqueous solution, and (b) acetic acid aqueous solution at liquid temperature of 180°C. Acid concentration is 2% v/v



## Results and Discussion

When ESI source was heated above room temperature, the protein in the solution denatured, and the unfolding of protein structure was indicated by the shift of major peaks to the higher charge states in the mass spectrum. This thermal denaturation effect was well reported in the literatures [17–19]. In the case of a small protein like ubiquitin, after a drastic shift of charge states at around 80°C, the charge state distribution did not show significant change even when the solution was heated to ~100°C [17, 18, 35]. Previously, we found that the thermally induced fragmentation could be observed when the solution temperature was raised above 140°C. Those fragments resemble the  $\gamma$ -ion and  $b$ -ion in the gas-phase dissociation such as that in collision induced dissociation (CID) [35]. However, a close verification of other peaks showed an abundance of hydrolysis products. The aqueous solution used in that work contained 1% acetic acid; thus it is definitely possible to induce the acidic hydrolysis that is assisted by the high temperature.

Here, we verify the result again by using 2% formic acid solution, which has recently become a standard reagent for the chemical digestion. The mass spectra of ubiquitin obtained at 180°C are shown in Figure 2a. Except for the peptide fragment

(1-18), all other hydrolysis products are due to the cleavage at the Asp sites. The result with acetic acid (Figure 2b) shows the hydrolysis at exactly the same cleavage sites, but in contrast to formic acid, there is also a presence of dehydrated species (e.g.,  $(M+nH-H_2O)^{n+}$ ).

Another online digestion measurement was done with myoglobin. In the hot acidic solution the myoglobin was in its apo-form, and the intact or dehydrated apo-myoglobin could be detected at temperature as high as 100°C. Figure 3 shows the mass spectra of myoglobin under different ESI capillary temperatures: 160, 180, and 220°C. As expected, at 160°C and above, the intact apo-myoglobin was not observed, and the mass spectra were dominated by the peptide peaks originating from the thermally induced/accelerated acidic hydrolysis (chemical digestion). These mass spectra appear to be more complicated than those of MALDI because the peptide fragments are multiply charged. Nevertheless, with Orbitrap-Exactive, the mass resolution was enough to distinguish their charge state, and nearly all the peaks were identified to be the Asp-selective hydrolysis products.

A list of observed digestion products is shown in Table 1. The assignment of the amino acid sequence is based on the accurate measurement of isotopic mass with error smaller than

**Table 1.** Observed Digestion Products of Myoglobin Using the Online Chemical Digestion ESI with Formic Acid at Different Ion Source Temperatures

Position	Sequence	Observed ion		
		160°C	180°C	220°C
1-43	GLSDGEWQQVLNVWGKVEADIAGHGQEVLR FTGHPETLEKF	$[M+4H-H_2O]^{4+}$	$[M+4H-H_2O]^{4+}$	
1-44	GLSDGEWQQVLNVWGKVEADIAGHGQEVLR FTGHPETLEKFD	4+, $[M+4H-H_2O]^{4+}$	$[M+4H-H_2O]^{4+}$	
4-43 /5-44	(D)GEWQQVLNVWGKVEADIAGHGQEVLR LFTGHPETLEKF(D)	4+, 5+	4+, 5+	
4-59 /5-60	(D)GEWQQVLNVWGKVEADIAGHGQEVLR LFTGHPETLEKFDKFKHLKTEAEMKASE(D)	5+, 6+, 7+	5+, 6+, 7+	
5-43	GEWQQVLNVWGKVEADIAGHGQEVLR LFTGH PETLEKF	4+, 5+	4+, 5+	$[M+nH-H_2O]^{n+}$ $n=4, 5$
5-59	GEWQQVLNVWGKVEADIAGHGQEVLR LFTGH PETLEKFDKFKHLKTEAEMKASE	6+	5+, 6+, 7+	
21-43 60-108 /61-109	IAGHGQEVLR LFTGHPETLEKF (D)LKKHGTVVLTALGGILKKKGHHEAELKPLA QSHATKHKIPIKYLEFIS(D)	7+	3+, 4+ 7+	3+, 4+
61-108	LKKHGTVVLTALGGILKKKGHHEAELKPLA QSHATKHKIPIKYLEFIS	8+	5+, 6+, 7+, 8+	$[M+nH+mCO]^{n+}$ $n=5-7, m=0, 1$
60-121 /61-122	(D)LKKHGTVVLTALGGILKKKGHHEAELKPLA QSHATKHKIPIKYLEFISDAIIHVLHSHKHPG(D)		7+, 8+	
61-121	LKKHGTVVLTALGGILKKKGHHEAELKPLA QSHATKHKIPIKYLEFISDAIIHVLHSHKHPG		7+, 8+, 9+, 10+	$[M+nH-H_2O]^{n+}$ $n=7-10$
60-125	(D)LKKHGTVVLTALGGILKKKGHHEAELKPLA QSHATKHKIPIKYLEFISDAIIHVLHSHKHPGDFGA(D)	9+		
61-125	LKKHGTVVLTALGGILKKKGHHEAELKPLA QSHATKHKIPIKYLEFISDAIIHVLHSHKHPGDFGA		9+	
110-153	AIHVLHSHKHPGDFGADAQGAMTKALE LFRNDIAAKYKELGFQ	5+, 6+, 7+	5+, 6+	
123-153 127-153	FGADAQGAMTKALELFRNDIAAKYKELGFQ AQAQAMTKALELFRNDIAAKYKELGFQ	3+, 4+ 3+, 4+	3+, 4+ 3+, 4+	
142-153	IAAKYKELGFQ	2+	2+	2+

The aspartate residues (D) are bolded

$n+$  refers to the protonated peptide,  $[M+nH]^{n+}$

Amino sequence is assigned by accurate measurement of their monoisotopic masses

Observed  $m/z$  and calculated values are listed in Supplementary Table T1



10ppm compared with the calculated values. A list of detected and calculated  $m/z$  is tabulated in Supplementary Table S1. The absence of Asp in some digestion products is due to the double cleavage event at both C-terminal of Asp and N-terminal of Asp. Those species are quite common in the chemical digestion, particularly with higher temperature or longer heating period [5, 7].

Both measurements with 160 and 180°C gave 100% sequence coverage of myoglobin (apo), but in the case of 160°C, one target Asp site at position 20 was not cleaved. At 180°C, however, all Asp sites were cleaved. Under excessive temperature at 220°C, the number of detected peak decreased and the dominant peaks were from the peptide fragment (61-108) in the form of  $(M+nH+mCO)^{m+}$ , where  $m=0, 1$ . This addition of 28 mass unit is due to the formylation, which is known to take place during formic acid chemical digestion [5]. The results with acetic acid solution at temperature 180°C and 220°C are depicted in Supplementary Figure S5a and b. Acetic acid produced nearly the same peptide fragments as that of formic acid at 180°C, but similar to the results of ubiquitin, there is a significant abundance of dehydrated species. At 220°C, formylation was not observed and the dominant peaks originate from the peptide (60-108) with the loss of two  $H_2O$ .

Although the dehydrated species were found to be more abundant in acetic acid, they were not completely absent in formic acid. Earlier literature that used formic acid and acetic acid for Asp-selective digestion attributed those peaks to the formation of pyroglutamate at N-terminal glutamine [5, 7]. Although we used a different heating approach (higher temperature but much shorter heating time), origins of the dehydration processes were likely to be similar. The acidity of acetic acid solution (pH2.5–3) at 1–2% (v/v) was weaker than that of formic acid (pH1.9) at the same concentration, but the results were similar when we repeated the digestion measurement with 10% (v/v) acetic acid (pH2.0). The formation of pyroglutamate appeared to be independent of acid type used, but it took place more easily in acetic solution at relatively lower temperature than in formic acid.

An inherent characteristic of Asp-specific digestion is that it produces peptides that are, on average, heavier than tryptic peptides, and they are multiply charged when ionized with electrospray. Standard proteomic workflow may need to be modified to accommodate this digestion method. By using LC-ESI-LTQ-Orbitrap and acetic acid digestion, Swatkoski et al. showed that database search with CID MS/MS (for small peptide) was successful with some modification on the MAS-COT enzyme rules [6]. For larger and highly charged Asp-cleavage peptides, ETD (and ECD) was found by Hauser et al. to be more suitable than CID to yield larger sequence coverage [37].

## Conclusion

The results here show that the selective acidic hydrolysis (chemical digestion) can be done rapidly by simply infusing

the peptide/protein solution through the superheated electrospray ion source. Both formic acid and acetic acid show the selective Asp-specific cleavage, and a temperature  $\sim 180^\circ\text{C}$  was enough to provide the coverage for all Asp sites for ubiquitin and myoglobin. The hydrolysis process was presumably taking place inside the heated stainless steel capillary of the ion source, and the peptide fragments were ionized in situ when the solution reached the tip of the ESI emitter. The whole processes of digestion, ionization, and MS acquisition was done in less than one-half min. In addition to hydrolysis, the present method can also be used to study the denaturation of protein or DNA and the analysis of protein complexes in a temperature range that cannot be achieved under atmospheric pressure.

Although the study here was conducted with aqueous solution, the super-heated electrospray had also been tested to be stable for organic solvent mixture and even neat methanol up to 180°C. The reported temperatures that correspond to a vapor pressure of 27atm for methanol and acetonitrile are  $\sim 180^\circ\text{C}$  and  $\sim 230^\circ\text{C}$ , respectively [38, 39]; thus, in principle, the superheated electrospray should be compatible with conventional or even superheated water eluent capillary liquid chromatography [27, 28]. Owing to the large i.d. (0.25mm) of the heated capillary used in the present prototype, it took more than 20s at  $4\mu\text{L}/\text{min}$  for the liquid to travel from the inlet port to the emitter tip, and this may broaden the original LC peak width. Using narrower heating capillary with i.d., say, 0.1mm, will increase the heat transfer efficiency and reduce the liquid traveling time to several seconds at the same flowrate. Further work will be done to explore whether such a short time scale is enough for complete chemical digestion or not.

## Acknowledgment

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