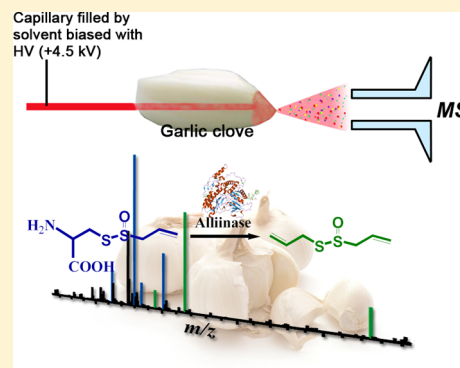# Molecular Characterization of Ongoing Enzymatic Reactions in Raw Garlic Cloves Using Extractive Electrospray Ionization Mass Spectrometry

Hua Zhang, Konstantin Chingin, Liang Zhu, and Huanwen Chen*

Jiangxi Key Laboratory for Mass Spectrometry and Instrumentation, East China Institute of Technology, Nanchang 330013 P.R. China

S *Supporting Information*

ABSTRACT: Characterization of enzymatic reactions occurring in untreated biological samples is of increasing interest. Herein, the chemical conversion of alliin to allicin, catalyzed by allinase, in raw garlic cloves has been followed in vivo by internal extractive electrospray ionization mass spectrometry (iEESI-MS). Both precursors and products of the enzymatic reaction were instantaneously extracted by infused solution running throughout the tissue and directly electrospray ionized on the edge of the bulk sample for online MS analysis. Compared to the room-temperature (+25 °C) scenario, the alliin conversion in garlic cloves decreased by (7.2 ± 1.4) times upon heating to +80 °C and by (5.9 ± 0.8) times upon cooling to −16 °C. Exposure of garlic to gentle ultrasound irradiation for 3 h accelerated the reaction by (1.2 ± 0.1) times. A 10 s microwave irradiation promoted alliin conversion by (1.6 ± 0.4) times, but longer exposure to microwave irradiation (90 s) slowed the reaction by (28.5 ± 7.5) times compared to the reference analysis. This method has been further employed to monitor the germination process of garlic. These data revealed that over a 2 day garlic sprouting, the allicin/alliin ratio increased by (2.2 ± 0.5) times, and the averaged degree of polymerization for the detected oligosaccharides/polysaccharides decreased from 11.6 to 9.4. Overall, these findings suggest the potential use of iEESI-MS for in vivo studies of enzymatic reactions in native biological matrices.

Capillary filled by solvent biased with HV (+4.5 kV)
Garlic clove
MS
Alliinase
m/z

Chemical characterization of enzymatic reactions in living organisms is of paramount importance to reveal the molecular mechanisms of life processes.[1−4] Real-time monitoring of in vivo enzymatic reactions occurring inside biological tissues challenges many well-established analytical techniques, because complex biological matrices hinder direct access to the molecular information about the enzymatic reaction.[5] Currently, multistep sample pretreatment (e.g., smashing, centrifugation, extraction, and proteolysis, etc.) is routinely required to study enzymatic reactions offline.[6−8] With sample pretreatments, a variety of techniques can be used for the analysis of enzyme properties, including optical spectrophotometry,[9] electrophoresis,[10] affinity chromatography,[11] X-ray crystallography,[12] proteomic sequencing,[13] and mass spectrometry.[14,15] Advanced mathematical models and data processing are also used to predict the enzymatic activities in the native biological environment using in vitro sample analysis.[16−18] For example, the activity of alliinase in garlic (*Allium sativum*) is traditionally studied in the biologically isolated environment using offline approaches.[19−21] However, the biological relevance of enzymatic properties obtained by offline methods remains unclear, because some part of important biochemistry information may be lost during the enzyme purification process or due to poor enzyme immobilization.[5] Clearly, in vivo

measurements would be extremely useful for chemical characterization of enzymatic reactions in living organisms.

Powered by the advent of direct ionization techniques such as desorption electrospray ionization (DESI),[22] direct analysis in real time (DART),[23] laser ablation electrospray ionization (LAESI),[24] low-temperature plasma probe (LTP),[25] neutral desorption extractive electrospray ionization (ND-EESI),[26] paper spray (PS),[27] and leaf spray ionization,[28] among others, ambient mass spectrometry (MS) has been increasingly used for the molecular analysis of complex raw samples in multiple disciplines.[29−32] Direct detection of trace analytes on various surfaces or inside biological liquids was successfully realized using ambient mass spectrometry.[33−35] Mass spectrometric analysis of the ongoing enzymatic reaction inside intact biological tissues with no/minimal perturbation to the native conditions is highly desirable.[36,37]

Earlier reports show that iEESI-MS has the unique capability to chemically profile plant tissues without seriously affecting tissue integrity.[38,39] Here we applied iEESI-MS to characterize the alliin conversion reaction catalyzed by allinase in raw garlic bulbs. In intact garlic cells, alliin is localized in the cell

cytoplasm, whereas alliinase is compartmentalized in vacuoles, so that these two molecules are physically separated. The reaction is initiated when intact cells are ruptured or wounded to allow release of the enzyme into cytoplasm.[40,41] By passing high voltage biased organic solvent through the garlic tissue, local membrane ruptures would be implemented, so that the reaction products could be produced, released, and observed in real time. The key organosulfur compounds (e.g., allicin) generated during the alliin conversion process were instantaneously detected, showing the dynamic response of the enzymatic reaction to various environmental factors, including high/low temperature, microwave/ultrasound irradiation, and extraction solution of different pH values. This method was also applied to in vivo profiling of the metabolites generated inside a sprouted garlic clove. The experimental data suggest that iEESI-MS might be a valuable analytical platform for in vivo studies of ongoing enzymatic reactions occurring in untreated bulk biological samples.

■ METHODS

iEESI-MS Analysis. The iEESI-MS experiments were implemented on an LTQ mass spectrometer (LTQ-XL, Finnigan, San Jose, CA) using a homemade iEESI source. To standardize the sample manipulation for better comparison, garlic tissue samples were cut to form a triangular shape with the thickness of ~3 mm and the side length of ~10 mm, as described elsewhere.[39] A fused silica capillary (ID 0.10 mm, OD 0.15 mm, Agilent Technologies Co., Ltd., U.S.A.) was inserted into the sample, allowing a distance of 2 mm between the capillary tip and the sample apex. The apex of the sample was pointed to the ion entrance of the mass spectrometer, with a distance of 5 mm (Figure S1). The extraction solutions (e.g., methanol, ammonium acetate aqueous solution) biased with a high voltage (+4.5 kV) was injected through the bulk garlic tissue at a flow rate of 2 μL/min, and the produced fine charged droplets containing analytes were brought toward the mass spectrometer inlet. Precursor ions were isolated with a mass-to-charge ratio window width of 1.5 Da, and collision-induced dissociation (CID) was performed at 10−30% units of collision energy. The temperature of the LTQ capillary was 150 °C. Other parameters were set as the default values of the instrument, and no further optimization was performed.

Materials and Reagents. Garlic cloves were purchased from a local supermarket. Garlic cloves with similar size and shape were carefully selected. Ultrasound irradiation was carried out in an ultrasonic bath (150 W/2450 Hz, KQ3200B, Jiangsu Kunshan Ultrasonic Equipment Tech. Corp. Ltd., Jiangsu, China) for 3 h. Stable temperature during sonication (25 °C ± 0.5 °C) was controlled by a thermostatic setup. In the case of cooling stimuli, garlic cloves were stored in the refrigerator (−16 °C) for 3 h and then warmed up to room temperature for iEESI-MS analysis. To perform the high-temperature stimuli experiment, the intact garlic cloves were soaked into 80 °C hot water for 10 min. Untreated cloves were used as a control group in all the cases. To study the microwave irradiation effect, three groups of garlic cloves were irradiated using a microwave oven (350 W, 2450 Hz) for different periods (0, 10, and 90 s). Ammonium acetate solutions of different pH values (pH 2.2, 6.7, and 11.4), adjusted with either acetic acid or strong ammonia−water, were used as the iEESI extraction solvent. Raw garlic cloves were cultured for 2 days at 25 °C to yield green sprouts of 2−3 cm in length. All the garlic samples were analyzed directly without further preparation.

Methanol and acetic acid were both of HPLC grade and bought from ROE Scientific Inc. (Newark, U.S.A.). Ammonium acetate and ammonium hydroxide were purchased at the highest grade available from Sinopharm Chemical Reagent Co. Ltd. (Shanghai). The deionized water used for the experiments was provided by ECIT chemistry facility.

■ RESULTS AND DISCUSSION

iEESI-MS of Alliin Conversion Reaction in Untreated Garlic Samples. MS studies of garlic commonly rely on GC and/or HPLC separation.[41−43] However, the chemical instability and thermal liability of thiosulfinates such as alliin and allicin normally requires derivatization step prior to conventional GC and HPLC interrogations. In contrast, molecular features are easily accessible by iEESI-MS without significant disturbance to the ongoing enzymatic reactions inside living tissues. Figure 1 shows the iEESI mass spectra of metabolites inside the garlic tissue recorded in the mass range m/z 50−400 in a positive ion detection mode. Due to the in situ extraction/ionization of the tissue sample, ionic inorganic species present in garlic tissue, such as Na^+, K^+, NH_4^+, acted as cationic reagents during the ionization process. This gives rise to $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, and $[M + NH_4]^+$ types of analyte ions. A variety of chemical constituents such as amino acids (e.g., arginine (m/z 175, $[M + H]^+$), lysine (m/z 147, $[M + H]^+$), valine (m/z 118, $[M + H]^+$), and leucine (m/z 132, $[M + H]^+$)), sugars (glucose (m/z 219, $[M + K]^+$) and sucrose (m/z 381, $[M + K]^+$)), alkaloid (choline (m/z 104, M^+)) and organosulfur compounds (e.g., alliin, allicin) were successfully detected and identified based on the collision-induced dissociation (CID) experiments (Figure S2) and previously reported data.[39,44,45] The instantaneous observation of both the precursors and products of enzymatic reaction allows direct qualitative evaluation of the reaction. Note that the precursor chemicals of interest, alliin (MW 177) and one of the major products, allicin (MW 162), were fingerprinted at m/z 178 [alliin + H]$^+$, m/z 216 [alliin + K]$^+$, m/z 393 [2(alliin) + K]$^+$, m/z 163 [allicin + H]$^+$, m/z 180 [allicin + NH$_4$]$^+$, m/z 185 [allicin + Na]$^+$, m/z 201 [allicin + K]$^+$, m/z 325 [2(allicin) + H]$^+$, and m/z 363 [2(allicin) + K]$^+$, respectively (Figure 1a). It is the sum of all pertinent peaks such as $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, and $[M + NH_4]^+$ that is employed in the calculation of intensity ratios between allicin and alliin. It is worth noting that analyte signal intensity in MS depends on the solubility, polarity, proton affinity, and other analyte chemical properties. Therefore, the observed signal intensities are affected by chemical matrix and cannot be directly employed to evaluate the ratio between reaction products. This greatly complicates evaluation of the absolute conversion rate by iEESI-MS as well as by other MS methods. However, the matrix effects are much less severe for the evaluation of relative changes in reaction rate (e.g., due to irradiation or heat exposure) because the estimates are done based on the relative intensity changes of the same analytes. Herein, the relative intensity ratio between allicin and alliin serves as an indicator characterizing the alliin conversion reactions ongoing in the garlic tissues.

Response of Enzymatic Reaction to External Stimuli. External stimuli including ultrasound irradiation, low and high temperature were applied to intact garlic cloves prior to iEESI-MS analysis. The positive effect of ultrasound on the activity of purified alliinase has been earlier reported in vitro.[46] Direct iEESI-MS measurement of garlic post ultrasound treatment also revealed differences in the spectral patterns compared to the
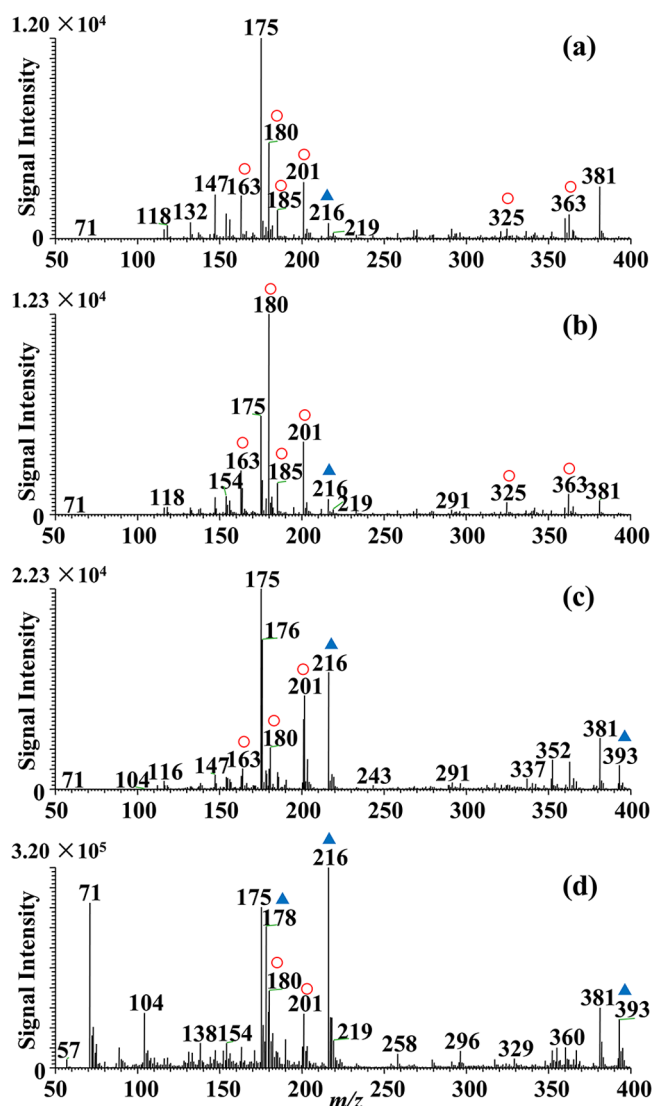
Figure 1. iEESI-MS spectra of garlic clove samples: (a) without external stimulation; (b) after exposure ultrasound irradiation (150 W/2450 Hz, 3 h), (c) after exposure to low temperature (−16 °C, 3 h), (d) after exposure to high temperature (80 °C, 10 min). The dominant peak assignments: m/z 71 ([$CH_3OH + K$]$^+$), m/z 118 ([valine + H]$^+$), m/z 132 ([leucine + H]$^+$), m/z 147 ([lysine + H]$^+$), m/z 163 ([allicin + H]$^+$), m/z 175 ([arginine + H]$^+$), m/z 178 ([alliin + H]$^+$), m/z 180 ([allicin + NH$_4$]$^+$), m/z 185 ([allicin + Na]$^+$), m/z 201 ([allicin + K]$^+$), m/z 216 ([alliin + K]$^+$), m/z 219 ([glucose + K]$^+$), m/z 325 ([2(allicin) + H]$^+$), m/z 363 ([2(allicin) + K]$^+$), m/z 381 ([sucrose + K]$^+$), m/z 393 ([2(alliin) + K]$^+$). Red ○ represents relevant allicin ions, and blue ▲ represents all alliin ions.

untreated cloves, especially for alliin and allicin. Higher intensity of allicin (e.g., peaks at m/z 180, 185, and 201) was recorded from the garlic clove stimulated by ultrasound (Figure 1b), whereas the signals of alliin (e.g., peaks at m/z 178, 216, and 393) dominated the mass spectra recorded from the garlic clove stimulated by temperature (Figure 1c,d). Alliin, a natural sulfoxide constituent in the cytoplasm of fresh garlic, is converted into allicin and other organosulfur compounds by alliinase within 10 s at 23 °C.[40,41] The different allicin/alliin ratio detected from the garlic samples treated with above-mentioned environmental stimuli indicates that the alliinase catalytic reactions were disturbed to certain extent. The alliin conversion was enhanced by (1.2 ± 0.1) times upon the

exposure of garlic to ultrasound irradiation for 3 h. Together with previous in vitro studies covering the ultrasound effect on the activity of purified alliinase,[46] our results suggest that gentle ultrasound irradiation applied to biological tissue can lead to a higher enzyme bioactivity.

High temperature is detrimental to fragile intermolecular network that shapes the active sites of an enzyme and can lead to the irreversible loss of the enzymatic property.[47,48] Our iEESI-MS measurement suggests that the alliin conversion in garlic was weakened by (7.2 ± 1.4) times after 10 min heating to 80 °C. Irreversibly weakened enzymatic catalytic ability was found in the iEESI-MS analysis of garlic cloves exposed to −16 °C for 3 h (Figure 1c), and a decrease factor of (5.9 ± 0.8) was derived. In contrast, we found that garlic cloves that had been cooled down to 4 °C restored enzymatic activity when gradually warmed up back to the room temperature. This curious example of alliinase being able to "hibernate" at mildly low temperature suggests the involvement of native biological matrix in protecting the function of naturally immobilized alliinase.[49,50]

Microwave Irradiation of Garlic Cloves. Microwave irradiation has been established as an efficient tool to accelerate chemical reactions.[51,52] In addition to the heating effects produced by microwave irradiation, the nonthermal effect of microwaves is attributed to the acceleration of enzymatic reaction.[51] It has been demonstrated that appropriately weak microwave irradiation poses additional catalytic effect to accelerate enzymatic reactions.[51] However, upon long irradiation the heating effects become more notable and ultimately result in irreversible damage of enzymes.[47] Here, selected garlic clove samples were exposed to microwave irradiation over different time periods (0, 10, and 90 s) and then directly analyzed by iEESI-MS. As shown in Figure 2, the iEESI-MS fingerprints of each garlic clove treated with microwave irradiation showed characteristic spectral patterns, which were distinctly different from the fingerprints of untreated cloves. Interestingly, higher intensity of allicin (peaks at m/z 180, 185, 201, and 362) was detected after 10 s microwave irradiation (Figure 2b), whereas lower intensity of allicin and higher intensity of alliin (peaks such as m/z 178, 216, and 393) were found after 90 s microwave exposure (Figure 2c). On the basis of signal ratio of allicin and alliin, we estimate that the alliin conversion was increased by (1.6 ± 0.4) times in response to a short period of microwave irradiation (10 s) prior to analysis. In contrast, (28.5 ± 7.5) times lower conversion was observed when garlic sample was exposed to microwave irradiation for 90 s before iEESI-MS. Overall, the iEESI-MS analysis of garlic cloves under different duration of microwave irradiation showed the same observations as earlier offline measurements, suggesting that alliinase activity in garlic tissue has high sensitivity to microwave irradiation treatment.

In Vivo Evaluation of the Enzymatic Reaction at Different pH Values. Although enzymes in living organisms are surrounded by advanced cellular matrix to maintain their activity,[53] severe alteration of the cellular/tissue environment might lead to the total or partial losses of enzymatic activity.[5,54] Here, ammonium acetate solutions with different pH values were infused through fresh garlic clove tissue to test if the enzymatic reactions responded to the associated changes in the pH of environment. Notable differences were observed in the mass spectra recorded from the garlic tissues treated with different pH (Figure 3). The peak at m/z 180, [allicin + NH$_4$]$^+$, dominates in the spectra for all the tested garlic cloves. The
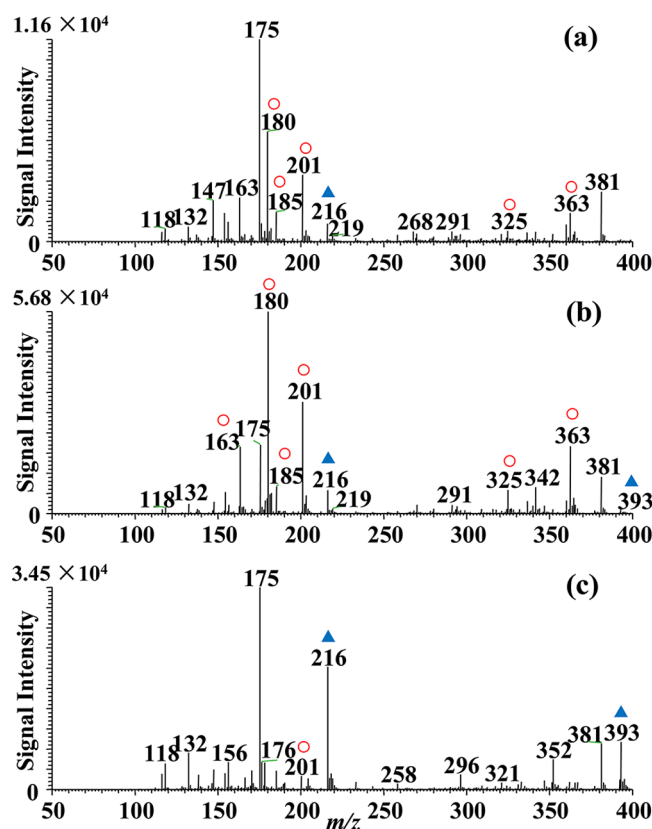
Figure 2. iEESI-MS spectra of garlic clove samples subjected to microwave irradiation at 350 W/2450 Hz: (a) 0 s, (b) 10 s, (c) 90 s. The dominant peak assignments: m/z 118 ([valine + H]$^+$), m/z 132 ([leucine + H]$^+$), m/z 147 ([lysine + H]$^+$), m/z 163 ([allicin + H]$^+$), m/z 175 ([arginine + H]$^+$), m/z 180 ([allicin + NH$_4$]$^+$), m/z 185 ([allicin + Na]$^+$), m/z 201 ([allicin + K]$^+$), m/z 216 ([alliin + K]$^+$), m/z 219 ([glucose + K]$^+$), m/z 325 ([2(allicin) + H]$^+$), m/z 363 ([2(allicin) + K]$^+$), m/z 381 ([sucrose + K]$^+$), m/z 393 ([2(alliin) + K]$^+$). Red ○ represents relevant allicin ions, and blue ▲ represents all alliin ions.

Figure 3. iEESI-MS spectra of garlic tissue samples obtained using extraction solution (100 mM NH$_4$OAc) with different pH values: a) pH 6.7, b) pH 2.2, b) pH 11.4. The dominant peak assignments: m/z 147 ([lysine + H]$^+$), m/z 163 ([allicin + H]$^+$), m/z 175 ([arginine + H]$^+$), m/z 178 ([alliin + H]$^+$), m/z 180 ([allicin + NH$_4$]$^+$), m/z 185 ([allicin + Na]$^+$), m/z 201 ([allicin + K]$^+$), m/z 216 ([alliin + K]$^+$). Red ○ represents relevant allicin ions, and blue ▲ represents all alliin ions.

richer chemical content of spectra recorded at pH 2.2 (Figure 3b) is believed to originate from higher extraction efficiency. Acidic environment promotes local ruptures of cell walls and thereby increases substance penetration through the cell wall.[55,56] Previous literature reported only slight variations of [M + H]$^+$ signal as a function of pH in the range of 3−11, even for nonamphoteric molecules.[57] Therefore, the higher signal intensity of alliin (m/z 216) at pH 11.4 (Figure 3c), is mainly attributed to the metabolic change rather than to the pH-induced change in ionization efficiency. As described in the Supporting Information (Figure S3), the <1 min period prior to the establishment of a stable signal represents a good example of the allicin/alliin ratio transition when the pH value was gradually changed from inherently neutral (∼7) to 2.2. The observation of strong alliin signals under extreme pH conditions suggests that in such an environment the bioactivity of alliinase is partially disabled. The reversibility of such pH-induced enzyme inhibition is of interest for further research.

Monitoring the Growth Stages of Garlic Cloves. The potential application of iEESI-MS for direct assessment of enzymatic reaction in sprouted garlic cloves was also demonstrated. Striking differences were shown for the mass spectra obtained from the unsprouted garlic clove and the sprouted clove (Figure 4). In comparison with the unsprouted
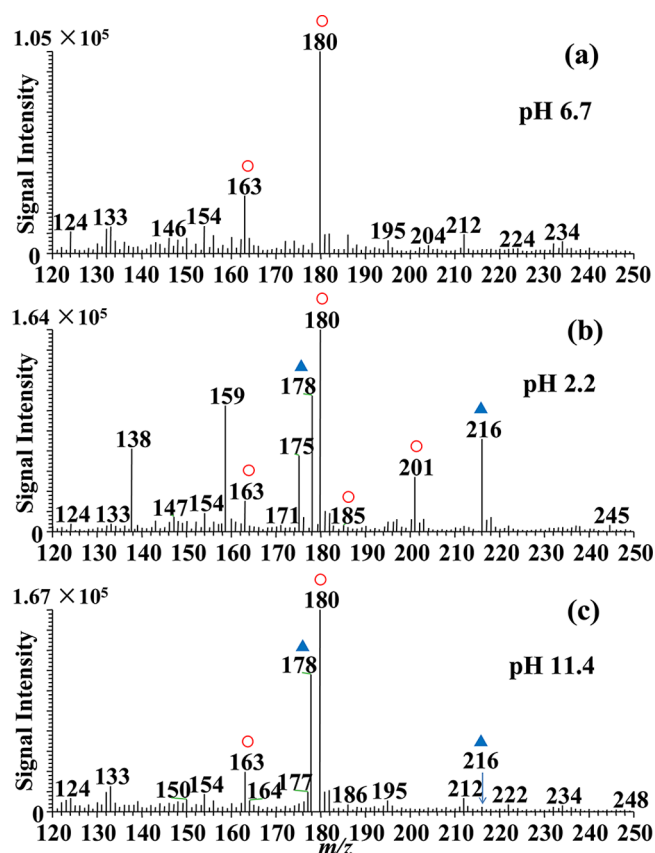
garlic samples, allicin signals (m/z 163, 180, and 201) were detected with increased intensity levels in the sprouted garlic clove (Figure 4b), indicating that the bioactivity of alliinase was enhanced in the sprouted garlic clove. The higher allinase activity in sprouted garlic clove is likely associated with the accelerated metabolism during the germination process.[58,59]

Apart from the small molecules, analytes of large molecular weight were also detected by iEESI-MS. For example, notable relative spectral differences were revealed in the mass range of m/z 500−4000 for the unsprouted (Figure 4b) and sprouted (Figure 4d) garlic cloves. The equidistant peaks with mass shift of 162 Da (m/z 543, 705, 867, 1029, 1191, 1353, 1515, 1677, 1839, 2001, 2163, 2325, 2487, 2649) were tentatively assigned to fructosans (($-$C$_6$H$_{10}$O$_5$$-$), 162 Da). This assignment is validated by the CID data. For example, the precursor ions of m/z 1515 generated major fragments of m/z 1353, 1191, 1029, 867, 705 in the CID spectrum (Figure S4). Other peaks notated in the full mass range spectra (Figure 4b,d) are consistent with previously reported data.[60,61] Interestingly, the concentration of saccharides in the sprouted garlic clove was found to be much higher than the concentration of saccharides in the unsprouted garlic clove, whereas the concentration of lipids (peak at m/z 758, 782) remained at the same level. This could be accounted by the fact that more saccharides, especially those of relatively low molecular weight, were produced in the
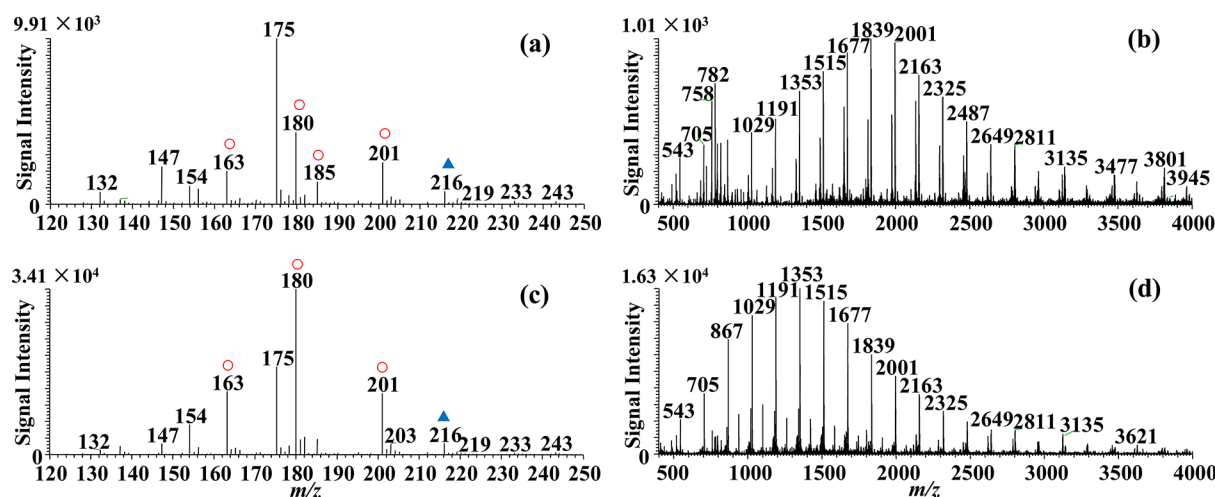
Figure 4. iEESI-MS spectra of garlic cloves at different growth stage: (a) unsprouted garlic clove (low mass range), (b) unsprouted garlic clove (high mass range), (c) sprouted garlic clove (low mass range), (d) sprouted garlic clove (high mass range). Red ○ represents relevant allicin ions, and blue ▲ represents all alliin ions.

garlic tissue in response to the enhanced respiration and material synthesis demands during the germination process.[58,62] The average degree of polymerization (DP) for the observed saccharides shifted from 11.6 (distributed in the range of 6−24) to 9.4 (distributed in the range of 3−18) during the germination process. In most cases, highly assembled saccharides (e.g., high DP polysaccharides and starch) in a living cellula cannot be directly utilized in the energy synthesis passage.[62] These polysaccharides are degraded to monosaccharides or disaccharides for metabolism synthesis in the garlic cloves. Normally, glycosyl hydrolases in the garlic cellula are responsible for converting large polysaccharides of high DP values to small polysaccharides/oligosaccharides of low DP values in the germination process.[58] The results summarized in Figure 4 demonstrate that iEESI-MS is able to measure simultaneous dynamic response for the broad molecular range of chemicals in a biological tissue and allows comprehensive characterization of long-term enzymatic conversions such as during the germination process of garlic cloves.

Sample Consumption. In the present study, the extracted sample size for one iEESI-MS experiment was around 1−3 mm^3 for the sake of easy operation. Given the usually high magnitude of product signals, we estimate that further reduction of physical sample size is principally feasible. It has been reported earlier that iEESI-MS allows detection of trace analytes at subppb level (0.0399 μg/L for salbutamol in pork tissue[38]). Therefore, the sensitivity should not be a major obstacle for the iEESI-MS of enzymatic reactions under optimized experimental conditions.

CONCLUSION

Chemical characterization of ongoing enzymatic reactions occurring inside living garlic samples has been demonstrated using internal extractive electrospray ionization mass spectrometry (iEESI-MS). Both large molecules (e.g., polysaccharides) and small molecules (e.g., amino acids, alkaloids, and organosulfur compounds, etc.) were successfully detected with high abundances from the interior of raw garlic cloves without sample pretreatment. The essential components (e.g., alliin, and allicin) involved in the enzymatic conversion of alliin were monitored in real time, showing that iEESI-MS is capable

to characterize the dynamic metabolic response to external stimuli including high and low temperature, microwave and ultrasound irradiation, and exposure to extreme pH values. The method established here has been further applied to molecular profiling of the germination process in garlic cloves, providing the metabolic profiles at different sprouting stages. The experimental results demonstrate that iEESI-MS can be a powerful method to probe the ongoing enzymatic reactions occurring in untreated biological samples and to allow deeper mechanistic understanding of enzyme properties in native biological environment.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: chw8868@gmail.com.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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