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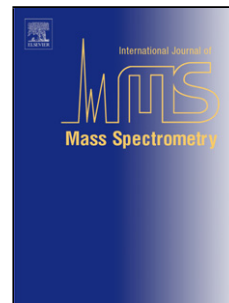
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Title: Detection of cysteine residue numbers in wheat gluten proteins by MALDI-TOF

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Highlights

The 4-vp treatment of protein can be used to measure the number of cysteine residue.

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Graphical abstract

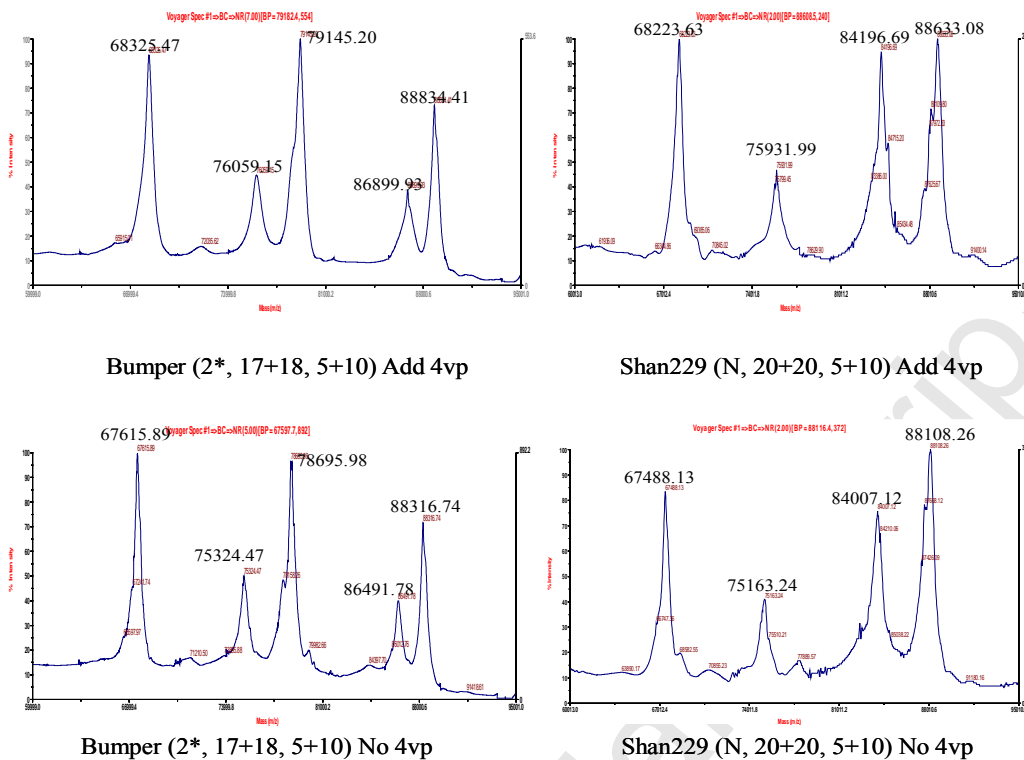


Fig. 1 The MALDI-TOF results of Bumper and Shan229 with 4-vp treatment and control, based on the changes of molecular mass value, the number of cysteine residue can be determined.

Detection of cysteine residue numbers in wheat gluten proteins**by MALDI-TOF**Ke Wang^{1,2,5}, Junhong Ma¹, Shahidul Islam^{1,3}, Yueming Yan², Rudi Appels¹, , GuijunYan⁴, Wujun Ma^{1,3*}

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Abstract:

The wheat seed storage protein plays a key role in determining processing quality. The disulfide bonds formed between cysteine residues in these proteins are critical in the formation of the unique rheological properties of wheat dough, which is the physical basis of bread making. Determining the number of cysteine residues in a particular protein, especially in high molecular weight glutenin subunits (HMW-GS) is an important task in evaluating wheat glutenin effects on end-product quality. In the current study, we established a fast method to accurately measure the number of cysteine residues in the HMW-GS. An alkylation reagent, 4-vinylpyridine (4-vp), was used to treat the proteins during extraction. For every cysteine residue in a protein, this treatment increases its molecular mass value by 105.14 Da, which can be accurately determined by MALDI-TOF equipment. Based on the changes of the molecular mass value caused by 4-vp treatment, the number of cysteine residue in a protein can be reliably determined. This method is also confirmed to be useful in studying non-glutenin proteins such as lupin seed storage proteins. It is expected that this method will speed up the process of selecting desirable HMW-GS in wheat breeding.

Keyword: Cysteine residue; Disulphide bonds; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF); 4-vinylpyridine; Wheat glutenin

Introduction

Most proteins in nature contain disulfide bonds between cysteine residues. These bonds are considered as structural motifs that were formed to help stabilize the tertiary or quaternary structure of proteins [1,2,3]. By stabilizing protein structure, disulfide bonds can protect proteins from damage and increase their half-life [4]. Moreover, they maintain protein integrity [4]. Cereal researchers have long been interested in disulphide bonds because of their role in determining the structure and functionality of wheat gluten proteins. Wheat storage protein, which consists of high molecular weight glutenin subunits (HMW-GS), low molecular weight glutenin subunits (LMW-GS), and gliadins [5], confer special visco-elasticity property that makes bread-making possible. The intra- and inter-molecular disulphide bonds in HMW-GS and LMW-GS facilitate the formation of glutenin polymer [6]. The size and composition of glutenin polymer are strongly correlated to flour properties [7]. Additional cysteine residue results in more cross-links with other glutenin subunits, resulting the formation of more elastic glutenin structure. For example, the HMW 1Dx5 subunit has an extra cysteine residue inserting a positive effect on dough elastic property [8]. Detecting the number of cysteine residues in a particular glutenin protein is of importance in predicting the effects of specific glutenin.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a high throughput tool for characterizing wheat gluten proteins [9,10,11,12,13,14,15,16,17]. Compared with the common separation methods, the MALDI-TOF-MS technique appears to be much more accurate and sensitive, requiring only few minutes per sample to perform the measurement [9]. Marchylo et al found that 4-vinylpyridine (4-vp) can combine with cysteine residue and stop the formation of disulphide bonds between cysteine residues [18]. The reaction between 4-vp and cysteine residue are often used for alkylation of protein prior to proteolytic

digestion, SDS-PAGE and spectra analysis [19]. In addition, the reaction introduces a positive charge to the target protein. In this study, by combining the 4-vp and cysteine reaction and the precision and sensitivity of MALDI-TOF-MS, we established a procedure to reliably detect the number of cysteine residues in gluten subunits. The application was also found useful in determining other types of proteins.

Material and methods

Plant materials

Twelve Australian cultivars, Ajana, Banks, Bullaring, Bumper, Calingiri, Chara, EGA Blanco, Endure, Pugsley, Halberd, IGW2944, and IGW3240, and two Chinese cultivars, Shan 229, Wanmai 33, were used in this study. Apart from these wheat lines, a Western Australian lupin cultivar Kalya was also used in this study.

Wheat HMW-GS extraction

Proteins were extracted from whole meal wheat grain according to the sequential procedure described by Singh et al [20]. Wheat sample (20 mg) was treated with 1.0 ml 70% ethanol (v/v) at room temperature for 30 min. After centrifuging for 5 min at 10,000 g, the supernatants was discarded and 1.0 ml of 55% isopropanol (v/v) was added into the tube and incubated for 30 min at 65 °C, followed by centrifuging for 5 min at 10,000 g. These steps were repeated three times to completely remove gliadins. The final precipitate was added with 150 µl extraction buffer containing 50% isopropanol (v/v), 1 M Tris-HCl (pH8.0), and 1% fresh DTT. The pellets were mixed thoroughly and incubated in a 65 °C water bath for 30 min, followed by centrifuging for 10 min at 10,000 g. After centrifugation, 60 µl supernatants were collected and 40 µl cold (-20 °C) acetone were added and stored at -20 °C overnight. Once the HMW glutenin subunit samples with no 4-vinylpyridine (4-vp) were prepared, another extraction buffer (90 µl) that using 1.4% 4-vp (v/v) instead of DTT was added and incubated for another 30 min at 65 .

After 60 μ l supernatant was collected and 40 μ l cold (-20 °C) acetone were added, the HMW glutenin subunit samples with 4-vp were prepared. The HMW glutenin subunits were purified by precipitation as described previously [18].

Lupin seed protein extraction

Lupin seed protein was used to verify the suitability of the method under development. Two proportions of seed proteins were used, including salt soluble protein and water-soluble protein. The salt soluble protein was extracted from lupin flour based on Duranti et al [21] and Lampart-Szczapa [22]. In detail, the lupin flour samples were defatted by Hexane at 20:1 ratio [23] and the extraction buffer (0.5M NaCl) was added at the ratio of 15 ml/g. The mixture was stirred for 4 hours at 4 °C and the supernatant was collected by centrifugation at 10,000 g for 10 mins. For water-soluble protein extraction, the extraction buffer was pure water, and the rest procedures are exactly the same as the salt soluble protein extraction.

MALDI-TOF-MS

The dried mixtures of HMW-GS samples were dissolved in 60 μ l acetonitrile (ACN)/H₂O (v/v, 50:50) containing 0.05% v/v trifluoroacetic acid (TFA) for 1 hour. Sample preparation was carried out according to the dried droplet method [24] using sinapinic acid (SA) as matrix, which was prepared by dissolving SA in ACN/H₂O (50:50 v/v) with 0.05% v/v TFA at a concentration of 10 mg/ml. Sample/matrix solution mixture (1:14 v/v) of 2 μ l was deposited onto a 100-sample MALDI probe tip and dried at room temperature.

MALDI-TOF mass spectrometric experiments were carried out with a Voyager DE-PRO TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with UV nitrogen laser (337 nm). Human transferrin (79,549 Da) was used as external standard for m/z calibration. The instrument was used with the following parameters: laser intensity 2,500, mass range 65-95 kDa, acceleration voltage 25 kV,

plate voltage 92%, guide wire 0.3%, and delay time 1,000 ns. Ten spectra of fifty laser shots were collected in a random pattern of center biased over the sample spot, which were automatically accumulated to form the final spectrum.

Results

The MALDI-TOF results of wheat varieties Bumper and Shan229 were shown in Fig. 1. The results clearly showed that when the HMW-GS was added with 4-vinylpyridine (4-vp), the M_r of the y-type HMW-GS increased by about 700 Da, the 1Dx5 increased by about 517 Da, and the 1Bx20 increased by 190 Da. Based on the changes of M_r values, the y-type HMW-GS can be concluded to contain 7 cysteine residues while the 1Dx5 and 1Bx20 contain 5 and 2 residues, respectively. The results were well consistent with existing knowledge about HMW-GS that y-type HMW-GS, 1Dx5 and 1Bx20 contain 7, 5 and 2 cysteine residues, respectively [8, 25, 26].

In order to confirm the accuracy of this method, 17 cultivars with known HMW-GS compositions were used to detect their number of cysteine residues (Table. 1). Results revealed that the 4-vp treatment of HMW-GS increased the M_r s of the HMW-GS and the increased figure corresponded to the number of cysteine residues, one cysteine residue resulted in an increase of 105.14 Da. An individual measurement usually resulted in 90-115 Da increase for one cysteine residue. The average increase per cysteine residue measured in Glu-Ax, Bx, By, Dx, and Dy were 102.56, 100.42, 104.19, 103.92, 103.67 Da, respectively, which were slightly off the expected value due to common machine error within the acceptable range. In order to achieve the reliability in measuring, multiple MALDI-TOF analysis for measuring the mean value of a sample is recommended. The 17 cultivars contained most of the known HMW-GS. No outlier was detected.

To test the suitability of this method in analysing other proteins, lupin seed storage protein alpha conglutin was used to measure its cysteine residue number (Fig

2). The left panel shows that the alpha conglutin of 19 kDa had an increase of about 105 Da after 4-vp treatment, indicating this protein contains 1 cysteine residue, while the rest spectrum peaks did not change after the 4-vp treatment, indicating no cysteine residue in these proteins. Following the same rule, every protein shown at the right panel should contain 1 cysteine residue (Fig 2).

Discussion

In this study, 4-vinylpyridine (4-vp) was used to detect the number of cysteine residue in a protein by MALDI-TOF. The theory was that each cysteine residue would combine one 4-vp molecule and the molecular mass increases 105.14 Da (the 4-vp molecular mass); the total increase of molecular mass is determined by the number of cysteine residues. For example, a protein with 4 cysteine residues will increase its *Mr* by 420.56 Da. The x-type HMW-GS usually have 2, 4, or 5 cysteine residues, while most y-type subunit have 6 or 7 cysteine residues [8,27,28].

Since the number of cysteine residues in a HMW-GS well correspond to its effects on wheat processing quality [8], it is useful to accurately determine their cysteine residue number. This is particularly true in evaluating a newly discovered protein. Another usage of this method is to discover new HMW-GS since all known HMW-GSs have 2, 4, or 5 cysteine residues and a protein with cysteine residue number outside these three numbers will deemed to be a novel HMW-GS. The reported method measures the change of *Mr* value directly from its spectrum, which was proven to be reliable and straightforward.

MALDI-TOF-MS has been used to characterize LMW-GS [13,29]. It would be ideal if the 4-vp method can be used to measure the number of cysteine residues in LMW-GS, which typically contains about 8 cysteine residues. However, during LMW-GS extraction, it is essential for 4-vp treatment in order to prevent the formation of disulphide bonds between individual LMW-GS molecules. This makes

this method not suitable for LMW-GS cysteine residue measurement since it is not possible to compare 4-VP treatment vs non-4-VP treatment of LMW-GS. It is worth noting that the M_r value of LMW-GS measured by MALDI-TOF is usually exaggerated since the protein was pretreated by 4-VP prior to MALDI-TOF analysis. Usually, a LMW-GS subunit contains 8 cysteine residues, the M_r by MALDI-TOF is expected to be about 840 Da higher than its real M_r .

Recently, 4-VP was used to label wheat prolamins for more efficient identification of cysteine-containing peptides in enzymic prolamins digests by electrospray ionization - tandem mass spectrometry [30]. In the current study, the 4-VP treatment of protein is used to fast and reliably measure the number of cysteine residues in a particular protein. This application is valuable when the number of cysteine residue is of importance such as glutenin proteins, of which the effect is largely determined by the number of cysteine residues. It is expected that this method will speed up the process of selecting desirable HMW-GSs in wheat breeding.

Acknowledgments

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Table. 1 The results of determine the number of cysteine residues about 17 cultivars with known HMW-GS compositions

cultivar	subunit	Glu-1Ax				Glu-1Bx				Glu-1By				Glu-1Dx				Glu-1Dy			
		Add 4-vp	No 4-vp	Difference	Average	Add 4-vp	No 4-vp	Difference	Average	Add 4-vp	No 4-vp	Difference	Average	Add 4-vp	No 4-vp	Difference	Average	Add 4-vp	No 4-vp	Difference	Average
Ajana	2*, 17+18, 2+12	86827.35	86457.82	369.53	92.38	79082.78	78692.00	390.78	97.70	76003.16	75257.64	745.52	106.50	87608.91	87168.65	440.26	110.07	69290.59	68550.76	739.83	105.69
Banks	2*, 7+8, 2+12	86913.13	86473.42	439.71	109.93	83009.38	82575.52	433.86	108.47	75954.27	75232.64	721.63	103.09	87629.09	87209.9	419.19	104.80	69365.29	68637.17	728.12	104.02
Bullaring	2*, 7+8, 2+12	86956.37	86491.7	464.67	116.17	83058.61	82651.97	406.64	101.66	75956.94	75271.21	685.73	97.96	87659.20	87278.03	381.17	95.29	69356.46	68661.14	695.32	99.33
Bumper	2*, 17+18, 5+10	86899.93	86491.78	408.15	102.04	79145.20	78695.98	449.22	112.30	76059.15	75324.47	734.68	104.95	88834.41	88316.74	517.67	103.53	68325.47	67615.89	709.58	101.37
Calingiri	N, 13+16, 2+12					83675.33	83245.75	429.58	107.40	77982.08	77220.82	761.26	108.75	87747.72	87301.1	446.62	111.66	69376.19	68652.79	723.4	103.34
Chara	2*, 7 ^{nc} +8, 2+12	86936.83	86540.16	396.67	99.17	83676.07	83233.1	442.97	110.74	75979.74	75259.79	719.95	102.85	87693.57	87258.45	435.12	108.78	69390.42	68656.89	733.53	104.79
EGA Blanco	2*, 7+9, 5+10	86876.2	86482.85	393.35	98.33	82978.92	82622.63	356.29	89.07	74302.77	73618.3	684.47	97.78	88794.28	88275.98	518.3	103.66	68287.19	67592.71	694.48	99.21
Endure	2*, 7+9, 5+10	86850.49	86443.63	406.86	101.72	82991.71	82604.73	386.98	96.75	74301.47	73583.41	718.06	102.58	88759.08	88242.55	516.53	103.31	68260.78	67493.72	767.06	109.58
IGW2944	2*, 13+16, 2+12	86938.42	86540.03	398.39	99.60	83604.66	83249.30	355.36	88.84	77885.46	77170.59	714.87	102.12	87609.93	87245.75	364.18	91.05	69350.87	68675.85	675.02	96.43
IGW3240	2*, 13+16, 2+12	86814.95	86400.08	414.87	103.72	83576.65	83127.58	449.07	112.27	77956.07	77128.52	827.55	118.22	87548.21	87155.4	392.81	98.20	69208.76	68526.58	682.18	97.45
Pugsley	N, 7 ^{nc} +8, 5+10					83490.51	83139.51	351	87.75	75862.73	75069.23	793.5	113.36	88585.52	88060.87	524.65	104.93	68265.87	67497.5	768.37	109.78
M12	N, 7+9, 2+12					82950.39	82567.86	382.53	95.63	74245.12	73533.7	711.42	101.63	87571.49	87204.45	367.04	91.76	69312.09	68589.78	722.31	103.19
M25	N, 7+9, 2+12					82993.22	82540.82	452.40	113.10	74255.45	73538.6	716.85	102.41	87639.25	87185.16	454.09	113.52	69309.85	68570.65	739.20	105.60
M26	N, 7+9, 5+10					82926.03	82538.12	387.91	96.98	74245.23	73520.58	724.65	103.52	88715.47	88180.18	535.29	107.06	68234.46	67500.89	733.57	104.78
Shan 229	N, 20+20, 5+10					84196.69	84007.12	189.57	94.79	75931.99	75163.24	768.75	109.82	88633.08	88108.26	524.82	104.96	68223.63	67488.13	735.5	105.07
Wanmai 33	N, 7+8, 5+10					82954.32	82532.49	421.83	105.46	75895.4	75208.52	686.88	98.13	88667.25	88121.64	545.61	109.12	68224.00	67470.67	753.33	107.62
Halberd	N, 20+20, 5+10					84003.54	83837.26	176.28	88.14	75733.51	75050.53	682.98	97.57	88553.88	88009.51	524.37	104.87	68168.23	67415.78	752.45	107.49

* It is well known that Y-type HMW-GSs have 7 cysteine residues, and most X-type HMW-GSs have 4 except 1Dx5 and 1Bx20 with 5 and 2, respectively.

Average=difference/the number of cysteine residue

Legends:

Fig. 1 The MALDI-TOF results of Bumper and Shan229 with 4-vp treatment and control

Fig 2. The measured molecular mass of lupin seed proteins increases due to 4-vp treatment. The left panel shows salt soluble protein as extracted by 0.5 M NaCl (A is control and B is treated by 4-vp) and right panel shows water-soluble proteins (C is control and D is treated by 4vp).

Fig. 1

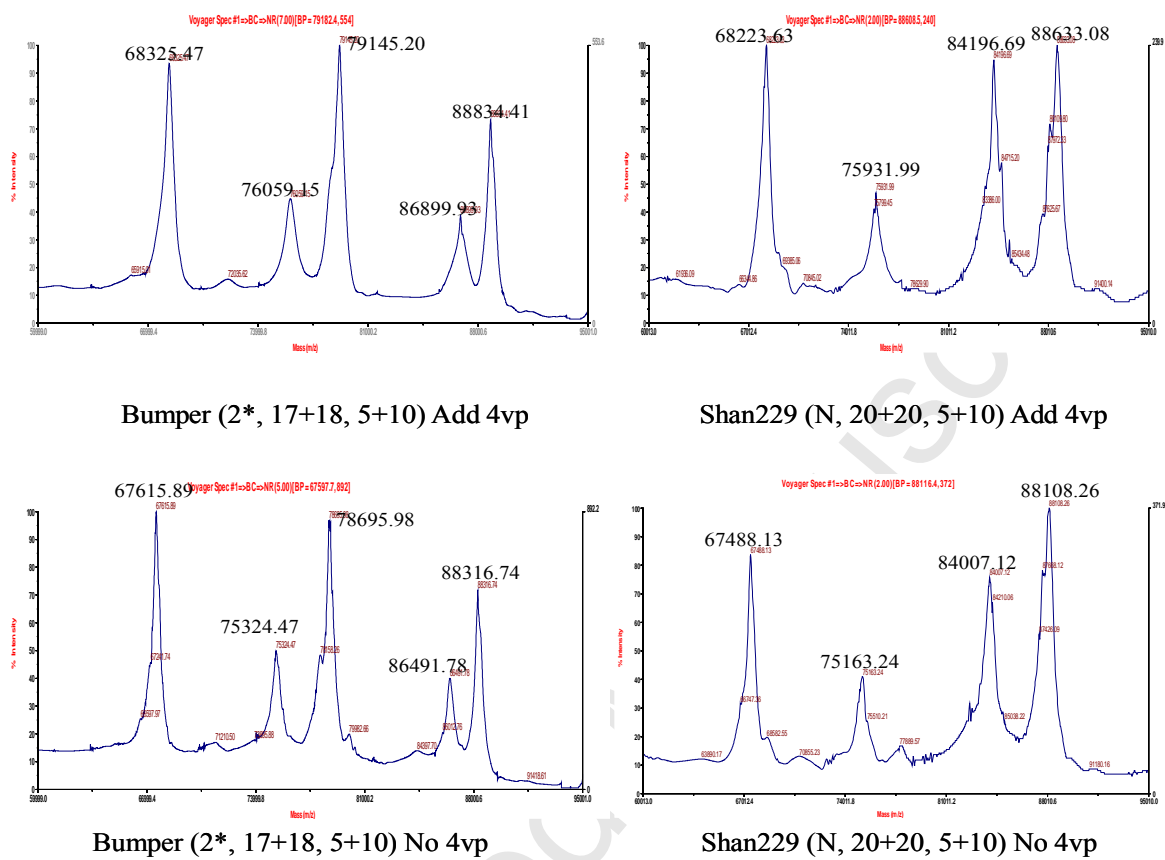


Fig. 2

