

Original article

Identification of four low molecular and water-soluble proteins from grape (*Vitis vinifera* L.) seeds

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Summary Profiles of soluble proteins isolated from mature seeds of grape (*Vitis vinifera* L.) pomace were studied using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). Two-dimensional gels stained with Coomassie brilliant blue revealed more than fifty protein spots. Four abundant protein spots showing low molecular weight (Mr) and wide isoelectric point (pI) were analysed by MALDI-TOF-MS, resulting in their identification. Taken together, these results suggest that identified proteins may be linked to seed development and metabolism, but more instructive is that they have some potential functions for future food application. These results provide some insights into conversion of grape processing wastes into useful products or even as raw material for other industries.

Keywords 2D electrophoresis, grape seeds, MALDI-TOF-MS.

Introduction

Because of the widespread demand for wine, table grapes and dried fruit, the common grape, *Vitis vinifera* L., is one of the most economically important fruit crops in the world. The global production of *V. vinifera* L. is around 69 million tons in 2006 (FAOSTAT, 2007). Since about 80% of the total amount is utilised for the production of wine and juice, ~10 million tons of grape pomace arise within a few weeks of the harvest time. Grape pomace contains 38–52% (on a dry matter basis) grape seeds (Schieber *et al.*, 2002), and the protein content of grape seed ranges from 10% to 13% (Fantozzi, 1981). As the amino acid composition and properties of proteins from grape seeds are very similar to those of cereals and other oilseeds, they could be used to substitute for these materials in foods where they are used as additives (Igartuburu *et al.*, 1991). However, numerous studies conducted during the last several decades on grape seeds only emphasised grape seeds extracts and procyanidins as well as their biological properties, for example, antioxidant properties and antiproliferative effects (Bucic-Kojic *et al.*, 2009; Choi & Lee, 2009). Protein composition and associated function of seeds from grape pomace during grape processing, remain largely unknown.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is one of the most powerful proteomics tools for the separation and quantification of proteins. Thousands of different proteins can be visualised in a 2D gel, allowing an overall analysis of the total content of proteins expressed in an organism. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), also widely established in proteomics, are sensitive methods for accurately characterising protein profiles. The identification of proteins can be performed by mass spectrometry which determines molecular masses with great accuracy (Herbert, 1999). A new method for differentiation of *V. vinifera* varieties by MALDI-MS analysis of the grape seed proteins has been developed. The water-soluble protein profiles of seed extracts from *V. vinifera* grape varieties were differentiated directly from their similarities and differences of MALDI spectra of the seed samples, with the proteins not identified (Pesavento *et al.*, 2008). MALDI-TOF-MS together with 2D-PAGE can be used both to visualise and compare complex mixtures of proteins and to gain a large amount of information about the individual proteins involved in specific biological responses (Adessi *et al.*, 1997; Gorg *et al.*, 2000; Finnie *et al.*, 2002). Hence, 2D-PAGE coupled with MALDI-TOF-MS has been used for grape proteome analysis in many studies. The first extensive proteomic study of ripe berry mesocarp from different grape genotypes was performed in 2004, which

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led to the identification of sixty-seven proteins, related to sugar or organic acid metabolism (Sarry *et al.*, 2004). The first proteomic analysis of grape skin has been performed by comparing three different ripening stages in grape berries (Deytieu *et al.*, 2007). Protein changes during grape berry ripening have been analysed by 2-DE and MALDI-TOF, in order to locate the differentially expressed proteins in berry development (Giribaldi *et al.*, 2007). The survey of tissue-specific protein expression patterns within pericarp tissues (skin and pulp) and seed tissues of grape berries from grapevines subjected to well-watered and water-deficit stress conditions has been performed recently (Grimplet *et al.*, 2009). The aim of these studies is to unravel the physiological events that characterise the grape berry ripening and the specific roles of different tissues at the protein levels, as well as some external conditions affected the events. However, there are few reports that focuses on grape seed representing one of the most important components from the grape pomace.

In the present study, 2-DE and MALDI-TOF were used to identify four most abundant water-soluble proteins in grape seeds, but not to perform a proteome analysis. The identification of these proteins (oxysterol-binding protein, Adenosine diphosphate(ADP)-glucose pyrophosphorylase, Nicotinamide adenine dinucleotide phosphate (NADP)-dependent sorbitol 6-phosphate dehydrogenase and Uridine diphosphate(UDP)-glycosyltransferase) is crucial for a better understanding of the many aspects related to grape seed development and metabolism, and then for evaluating their potential use for functional food.

Materials and methods

The extract preparation of total protein from *Vitis vinifera* L. seeds

Dry grape (*Vitis vinifera* L.) seeds separated from grape pomace were obtained from Tianjin Jianfeng Natural Product Co., Ltd. All seeds were passed through ten mesh sieve, and residual seed skin and stem were removed manually. Total protein was extracted as follows: About 1 kg of *V. vinifera* L. seeds was soaked in distilled water overnight and homogenised in four volumes of an aqueous buffer (50 mM phosphate buffer solution (PBS), 1% polyvinylpyrrolidone (PVP), pH 7.5) using a Waring blender. The slurry was centrifuged at 12 000 *g* for 15 min to separate the insoluble material. The supernatant was adjusted with a solution consisting of 7 M urea, 2 M thiourea, 2% 3-[(3-Cholanidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM 1,4-dithiothreitol (DTT) in the ratio 1/30 w/v under stirring at room temperature for 2 h. The slurry was centrifuged at 10 000 *g* for 30 min and the extracted proteins were immediately analysed or kept frozen at -80 °C until use.

2D Isoelectric focusing (IEF)/sodium dodecyl sulfate (SDS)-PAGE and image processing

Isoelectric focusing was performed on 18 cm, pH 3–10 nonlinear IPG strips (Amersham Pharmacia Biotech, Uppsala, Sweden). The strips were rehydrated overnight in a solution consisting of 8 M urea, 2 M thiourea, 0.02% CHAPS, 0.02 M 1,4-dithiothreitol (DTT) and 0.05% IPG buffer pH 3–10 (Amersham Pharmacia Biotech) containing the protein sample. For the total protein extract, an estimated amount of 800 µg of protein was loaded. These amounts were optimised for the best electrophoretic performance. Strips were focused at 80 000 Vh, with a maximum of 8000 V, at 20 °C using the IPGphor IEF System (Amersham Pharmacia Biotech). Prior to the second dimension, strips were incubated in equilibration buffer (1.5 M Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol) with 20 mM DTT for 15 min, and then with 100 mM iodoacetamide in the same buffer without DTT for 10 min. The separation was performed in 20 × 25 × 1 cm 13% acrylamide gels, containing 0.2% bisacrylamide, using a PROTEAN II xi Cell (BioRad, California, USA).

The running buffer consisted of 25 mM Tris-base, 192 mM glycine and 0.1% SDS. The electrophoresis conditions were as follows: 2D gel was firstly run at 40 mA for 40 min, and then run for 5 h at 60 mA at 25 °C. The gels were stained with Coomassie brilliant blue (CBB). Two-dimensional SDS-PAGE Standards (Amersham Pharmacia Biotech) were used as internal standards in selected gels for the evaluation of the relative molecular size and pI of the spots. To minimise experimental variability, two independent total protein extracts were obtained. The gels were digitalised in an Epson Expression 1680 Pro Scanner and analysed with ImageMaster 2D Platinum Software (Amersham Pharmacia Biotech). Master gel was generated for each sample by matching the available gels with the same software.

Protein in-gel digestion

Protein spots, around 1 mm², were cut from the 2D gel with a pipette tip and transferred to 1.5 mL microtubes, respectively. To each tube was added 100 µL destaining solution of 50 mM NH₄HCO₃ in 50% acetonitrile (ACN), and resultant mixture was incubated for 20 min. The solution was discarded. This process was repeated three times or until the blue color of the gel piece was completely removed. Then 200 µL of 100% ACN was added and incubated for 10 min. The gel piece changed from semi-transparent to opaque white. The solution was removed and the gel pieces were dried in a SpeedVac for 15 min. Subsequently, 5 µL of trypsin solution (0.005 µg µL⁻¹ in 25 mM NH₄HCO₃, pH 8.0) was added to each tube and incubated at 37 °C

overnight. The peptides were recovered from the gel pieces by incubating with 50 μ L of extraction solution of 50% ACN/0.1% trifluoroacetic acid (TFA) for 30 min. The extracted peptide solution was dried in the Speed-Vac to approximately 10 μ L and stored at -20°C for later use. Peptides were desalted and concentrated using ZipTip C18 columns before MS analysis.

Mass spectrometry analysis and protein identification

All mass spectra of MALDI-TOF-MS were obtained on a Bruker Ultraflex III TOF/TOF (Bruker Daltonik GmbH, Bremen, Germany) in positive ion mode at an accelerating voltage of 20 kV with a nitrogen laser (337 nm). The spectra were internally calibrated using trypsin autolysis products. The peptide sample was desalted with a spin column (Pierce, Thermo Fisher Scientific Inc, Rockford, USA) before MALDI-TOF-MS analysis. Approximately 0.5 μ L of sample and 0.5 μ L of matrix were spotted onto a stainless steel target plate and allowed to air-dry prior to analysis. The samples were ablated using ~ 100 laser shots fired in ten shot packets while the laser rastered over the target surface. All spectra were acquired in the positive ion reflectron mode using α -cyano-4-hydroxy cinnamic acid (CHCA) (10 mg mL^{-1} in 50% ACN/0.1% TFA) as the matrix.

Database search

Protein identification was performed by searching the NCBI nonredundant database using the Mascot search engine (<http://www.matrixscience.com>) where a probability-based scoring system was used. The following parameters were used for database searches with MALDI-TOF peptide mass fingerprinting data: type of search, sequence query; enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ± 100 ppm; fragment mass tolerance, ± 0.3 Da; max missed cleavages, 1; instrument type, MALDI-TOF-TOF. We introduced the MS signals corresponding to one of the unidentified 2D-electrophoresis spots (protein) into the Mascot analysis system. In order to obtain more information about the function of the identified protein, a BLAST search was carried out.

Results and Discussion

Extraction of grape seed proteins

Grape seeds have a total protein content of around 10–13% (Fantozzi, 1981). However, little is reported about the composition and property of proteins occurring in the grape seeds. Therefore, we carried out 2D-

PAGE analysis and firstly focused on the low-molecular and water-soluble proteins obtained by extraction of milled seeds in 50 mM PBS, pH 7.5, 1% PVP. Such buffer with comparatively low ionic strength were used on the basis of our own and others' experience with purification of specific proteins from dicotyledonous plant seeds, for example, ferritin, thaumatin-like proteins, nonspecific lipid transfer protein, esterase, phosphoglucosyltransferase, acid phosphatase, peroxidase, chitinases and alcohol dehydrogenase (Gianazza *et al.*, 1989; Laulhere *et al.*, 1989; Jean-Paul *et al.*, 1996; Tattersall *et al.*, 1997; Elide *et al.*, 2003; Zoccatelli *et al.*, 2007). In addition, the buffer used in the present study also minimised the extraction of storage proteins in order to prevent them from masking lower abundance proteins in the 2D gel electrophoresis profile. Thus, the extraction buffer used in this study likely enriched proteins and enzymes related to cellular metabolism and storage product.

Protein detection and identification

The proteins separated by 2D gel electrophoresis are subject to in-gel digestion with trypsin followed by MALDI-TOF-MS. The use of trypsin to digest the original protein in gel is a well-known procedure for efficient protein extraction prior to molecular MS analysis (Aebersold *et al.*, 1987). In Fig. 1, we illustrate an overall view of the 2D map obtained from the water-soluble components of the *V. vinifera* L. seeds

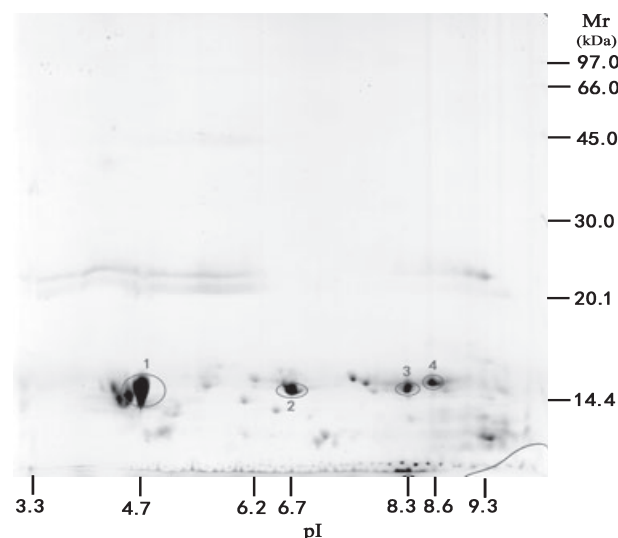


Figure 1 *Vitis vinifera* L. seed total protein 2D reference map. The spots analysed by trypsin-digestion and mass spectrometry are outlined and numbered. The full list of the identified spots is reported in Table 1. Gels and running conditions are detailed in materials and methods.

homogenate. Two-dimensional gels stained with CBB revealed more than fifty protein spots. The polypeptides profile of the proteins found in the seeds of grape (*V. vinifera* L.) appears simple with a predominance of some low-molecular weight polypeptides. The phenomenon is also observed in the juice of ripe grapes (Hsu & Heatherbell, 1987; Pueyo *et al.*, 1993). One possible reason for the simplicity of this profile is incomplete extraction of total cellular protein interfered with polyphenolic interaction. Another reason is that some polypeptides with molecular weight higher than 25.0 kDa cannot be visualised under the present experimental conditions. The horizontal streaks in the 2D pattern maybe imply globulin subunits with identical Mr (c. 23.0 kDa) into native aggregates of different pI (3.3–6.2) (Gianazza *et al.*, 1989). We selected four spots marked with numbers from 1 to 4 within small circles, which apparently corresponds to protein fragments of a wide pI and low subunit Mr, as shown in Fig. 1, in order to start the investigation of the nature of these proteins. Our interest in this area derived from the fact that these spots remained invariable from gel to gel repetitions and might correspond to specific proteins of *V. vinifera* L. seeds. Three spots (1–3) appear at around 14.4 kDa; spot 1 is the most intense. Spot 4 corresponds to proteins with the apparent subunit masses of approximately 15.0 kDa. The protein contained in each spot was submitted to in-gel trypsin digestion, and the mass of the resulting peptides was obtained from MALDI-TOF-MS analysis (Figs S1–S4). Mascot and BLAST analyses of these protein spots revealed clear similarities to proteins involved in seed development and metabolism.

Once we obtained the data from MALDI-TOF-MS analysis of spot 1 (Fig. S1), the most relevant MS signals

were introduced into the Mascot search engine (Lei *et al.*, 2005). Even if a protein fragment has a low molecular weight, the Mascot search of MS signals found a hit with a score higher than 71 ($P < 0.05$) for spot 1 (Table 1 and Fig. S1). The top score found was 74, correspondent to the protein gi|193848570 with 84 433 Da mass and described as oxysterol-binding protein (OSBP) (*Brachypodium distachyon*). Though there is the discrepancy between experimental weight for spot 1 and theoretical weight for the identified protein (Table 1), the identified protein is the best matched one through Mascot search with peptide matches as 13, so the identification is reliable (Lim *et al.*, 2003). Such difference most likely comes from their totally different plant resource. OSBP is the founding member of a multigene family of sterol-binding proteins implicated in lipid metabolism, vesicle transport, and signal transduction. Firstly, it is conserved in yeast, flies, worms, mammals and plants. These include mammalian OSBP, a protein of about 800 amino acid residues that binds a variety of oxysterols (oxygenated derivatives of cholesterol); yeast OSH1, a protein of 859 residues that also plays a role in ergosterol synthesis; and yeast proteins HES1 and KES1, highly related proteins of 434 residues that seem to play a role in ergosterol synthesis, PiORP1, a *Petunia* OSBP-related protein involved in receptor-kinase mediated signalling in pollen has also been identified (Li *et al.*, 2002). Secondly, OSBP are evolutionary related proteins involved with sterol synthesis and/or its regulation (Bussey *et al.*, 1994). They all contain a C-terminal oxysterol-binding domain (Huo *et al.*, 2009). Collectively, this shows that OSBP has a role in regulating lipid and sterol homeostasis, but its precise mechanism of action remains unknown. To date, no

Table 1 List of identified spots by MALDI-TOF-MS and software Mascot

Spot	Matched Protein	Accession number	Score	Gel molecular mass (Da)	Theoretical molecular mass (Da)	Gel pi	Theoretical pi	Peptide matches	Partial sequence
1	oxysterol-binding protein [Brachypodium distachyon]	gi 193848570	74	14740	84433	4.71	8.07	13	KKVSHHPMVMACHCE GRGWK
2	predicted protein [Physcomitrella patens subsp. patens]	gi 68004994	88	14593	232063	6.77	8.62	14	KQWSLQGGGAASPEEGELEDERM
3	NADP dependent sorbitol6-phosphate dehydrogenase [Amelanchier alnifolia]	gi 9310930	70	14839	29778	8.36	7.25	5	LQLDYLDLYLVHYMPMPTR
4	UDP-glycosyltransferase UGT85B2[Avena strigosa]	gi 87373028	49	15139	51303	8.68	5.34	5	ASSWYVNFSGSIHVMTAAQLR

Matched protein, in Mascot database;

Gel molecular mass/Gel pi, the molecular mass and pi on gel; Theoretical molecular mass/Gel pi, the molecular mass and pi calculated by Mascot databases.

OSBP correlative researches of *V. vinifera* L. seeds have been reported.

Protein spot 2 showed similarity to predicted protein from *Physcomitrella patens* ssp. *patens*. For the prediction of this protein function, we introduced the sequence of gi|168004994 into the BLAST analysis to get an initial idea of its function. Result suggested that ADP-glucose pyrophosphorylase (ADP-Glc PPase) (*Clostridium perfringens* SM101) was very similar to gi|168004994 previously identified using the Mascot server (Table 1). ADP-Glc PPase (EC 2.7.7.27) is a key enzyme in starch biosynthesis during photosynthesis in plants. It belongs to the family of transferases, specifically those transferring phosphorus-containing nucleotide groups (nucleotidyltransferases) (Ghosh & Preiss, 1966). This enzyme participates in starch and sucrose metabolism. It was first found in wheat flour by Espada (Espada, 1962). Subsequently, its occurrence in other nonphotosynthetic plant tissue was reported (Preiss *et al.*, 1967; Dickinson & Preiss, 1969). Considerable evidence indicates that ADP-Glc PPase is the rate-limiting enzyme of starch biosynthesis for photosynthetic tissues and several nonphotosynthetic tissues. Detailed characterisation of the sugar phosphate site and the 3D structure of *E. coli* ADP-Glc PPase, the Glc-1-P site have been reported (Bejar *et al.*, 2006). Previous studies indicated that there are relative abundant UDP-glucose pyrophosphorylase in grape berry, supporting a key role of the apoplastic pathway of sugar loading during berry ripening (Sarry *et al.*, 2004).

Protein spot 3 showed similarity to NADP-dependent sorbitol 6-phosphate dehydrogenase (S6PDH) from *Amelanchier alnifolia* (Table 1). This protein can catalyse an NADP-dependent oxidation of sorbitol-6-phosphate (S6P) to glucose-6-phosphate (G6P). The maximum velocity of the reduction of G6P was much higher than that of the oxidation of S6P and the enzyme had high G6P-reducing activity over the pH range from 7 to 11. The reduction of G6P to S6P was inhibited by the addition of nucleotide di- or triphosphates. Adenosine triphosphate (ATP), the strongest inhibitor, and ADP inhibited the reduction of G6P in a competitive manner with respect to NADPH (Kanayama & Yamaki, 1993). Furthermore, the level of S6PDH transcripts was higher in more developed plant tissues than in young tissues, and was concomitant to the alteration of enzyme activity and protein level. The S6PDH activity and protein level in plant tissues also varied seasonally. They were low in fruit maturation, and rose after fruit harvest. This change was probably controlled by the level of transcripts (Sakanishi *et al.*, 1998). Thus, it is suggested that S6PDH plays a major role in the biosynthesis of sorbitol from G6P during photosynthesis *in vivo*. Previous results suggest that the expression of the S6PDH gene is associated with source function. This protein has been related to plant metabolism, growth

and development, as well as to signalling regulate partitioning between sorbitol and glucose in plant tissues. S6PDH have been purified from apple seedlings, peach leaves, apple leaves and loquat leaves (Hirai, 1981; Yamaki, 1984; Kanayama & Yamaki, 1993; Sakanishi *et al.*, 1998). S6PDH from *V. vinifera* L. seeds was seldom reported although malic dehydrogenase isozymes, lactic dehydrogenase, glucose-6-phosphate 1-dehydrogenase and alcohol dehydrogenase activities of protein extracts from the endosperm of *V. vinifera* L. seeds have been detected by the methods of specific stains and zymograms (Gianazza *et al.*, 1989; Sarry *et al.*, 2004).

Protein spot 4 showed similarity to UDP-glycosyltransferase (UGT) (Table 1). Glycosyltransferases are enzymes (EC 2.4) that act as a catalyst for the transfer of a monosaccharide unit from an activated nucleotide sugar (also known as the 'glycosyl donor') to an glycosyl acceptor molecule, usually an alcohol (Egelund *et al.*, 2004). For UGT, the nucleotide sugar is UDP-glucose. Arabidopsis glycosyltransferases are capable of glucosylating coniferyl aldehyde and sinapyl aldehyde. In addition, the glucosyltransferase of transferring the glucose residue from UDP-glucose to the 4-OH- Phegly₄ residue of the vancomycin aglycone have been also reported (Breton & Imberty, 1999). UDP-glucose:protein transglucosylase has been identified during grape berry ripening (Giribaldi *et al.*, 2007).

In this study, oxysterol-binding protein (OSBP), ADP-glucose pyrophosphorylase (ADP-Glc PPase), NADP-dependent sorbitol 6-phosphate dehydrogenase (S6PDH) and UDP-glycosyltransferase (UGT) have been found in extracts of *V. vinifera* L. seeds. These proteins have been implicated in various functions. Previous studies indicated that OSBP plays a possible functional role in the lipid metabolism, atherosclerosis and hepatitis C virus (HCV) maturation processes (Olkkonen & Lehto, 2004; Amako *et al.*, 2009). Maybe it is tempting to speculate that OSBP could be exploited for the functional food for atherosclerosis prevention and HCV infection; sorbitol 6-phosphate dehydrogenase can be overexpressed in lactate dehydrogenase-deficient *Lactobacillus plantarum* to achieve nearly maximum yields of sorbitol from glucose, which is very rewarding in the context of sugar alcohols' more and more widely applications in pharmaceuticals, oral and personal care products, and as intermediates in chemical synthesis (Akinterinwa *et al.*, 2008). A UGT from apple root tissue can convert phloretin to phlorizin, a potent antioxidant in apple, and phlorizin contributes to the flavour, colour and health benefits of apple fruit and processed products (Jugd  *et al.*, 2008). Whether the UGT from *V. vinifera* L. seeds has the similar function is to be worth pursuing. However, to uncover these novel bioactive proteins and to reveal their real

functions or health benefits, future isolation and characterisation of these proteins is first-line.

Conclusions

We use 2D gel electrophoresis combined with MALDI-TOF mass spectrometry to identify four types of *V. vinifera* L. seed proteins, which are possibly involved in seed development and metabolism, by peptide mass fingerprinting. Their identification provides valuable information for understanding development of proteins in *V. vinifera* L. seed as dietary protein supplements. In future, our aim is to separate and purify these newly found proteins so as to be able to characterise their structure and function.

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Supporting Information

Additional supporting Information may be found in the online version of this article

Figure S1. The MALDI–TOF mass spectrum of protein spot 1.

Figure S2. The MALDI–TOF mass spectrum of protein spot 2.

Figure S3. The MALDI–TOF mass spectrum of protein spot 3.

Figure S4. The MALDI–TOF mass spectrum of protein spot 4.

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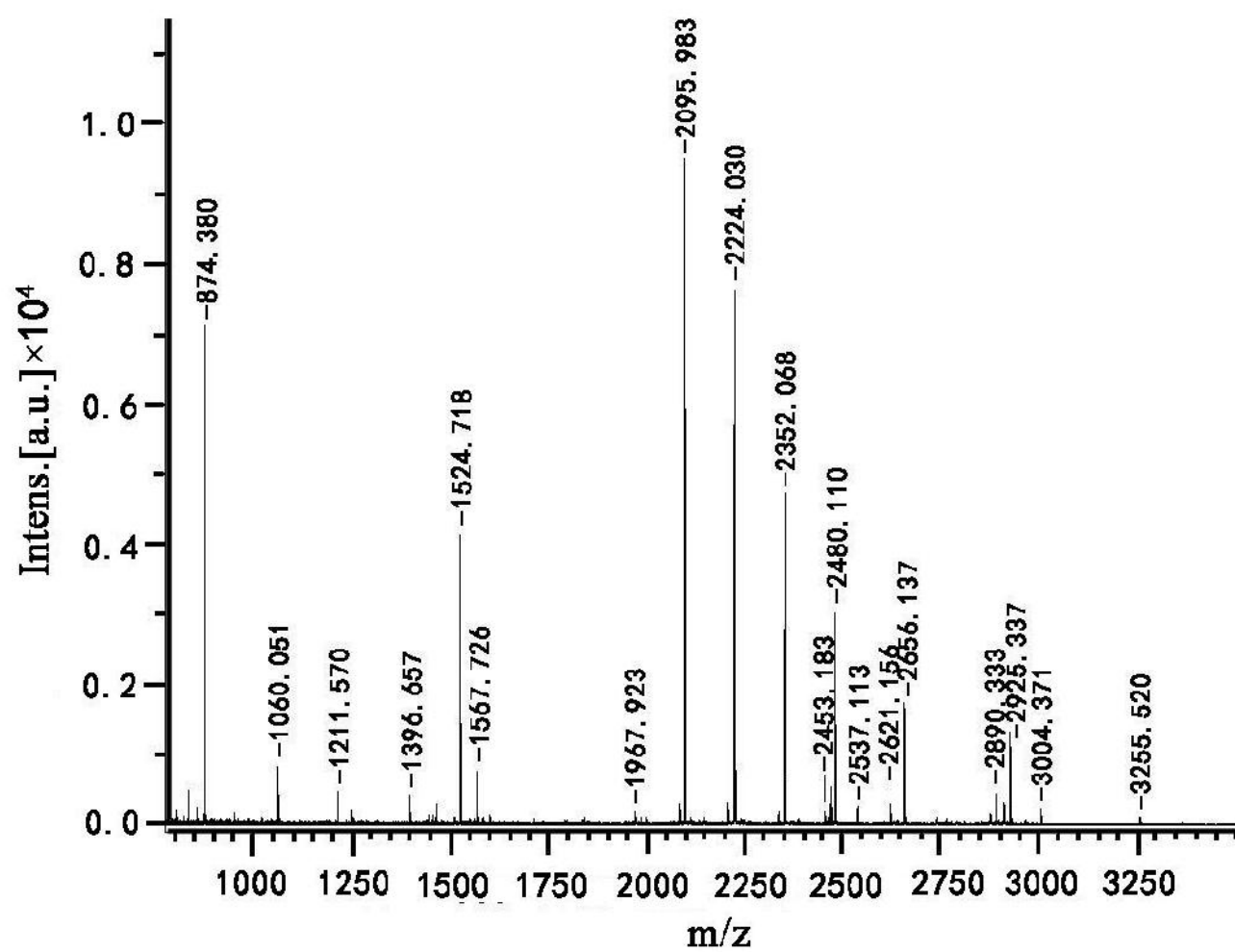


Figure S1

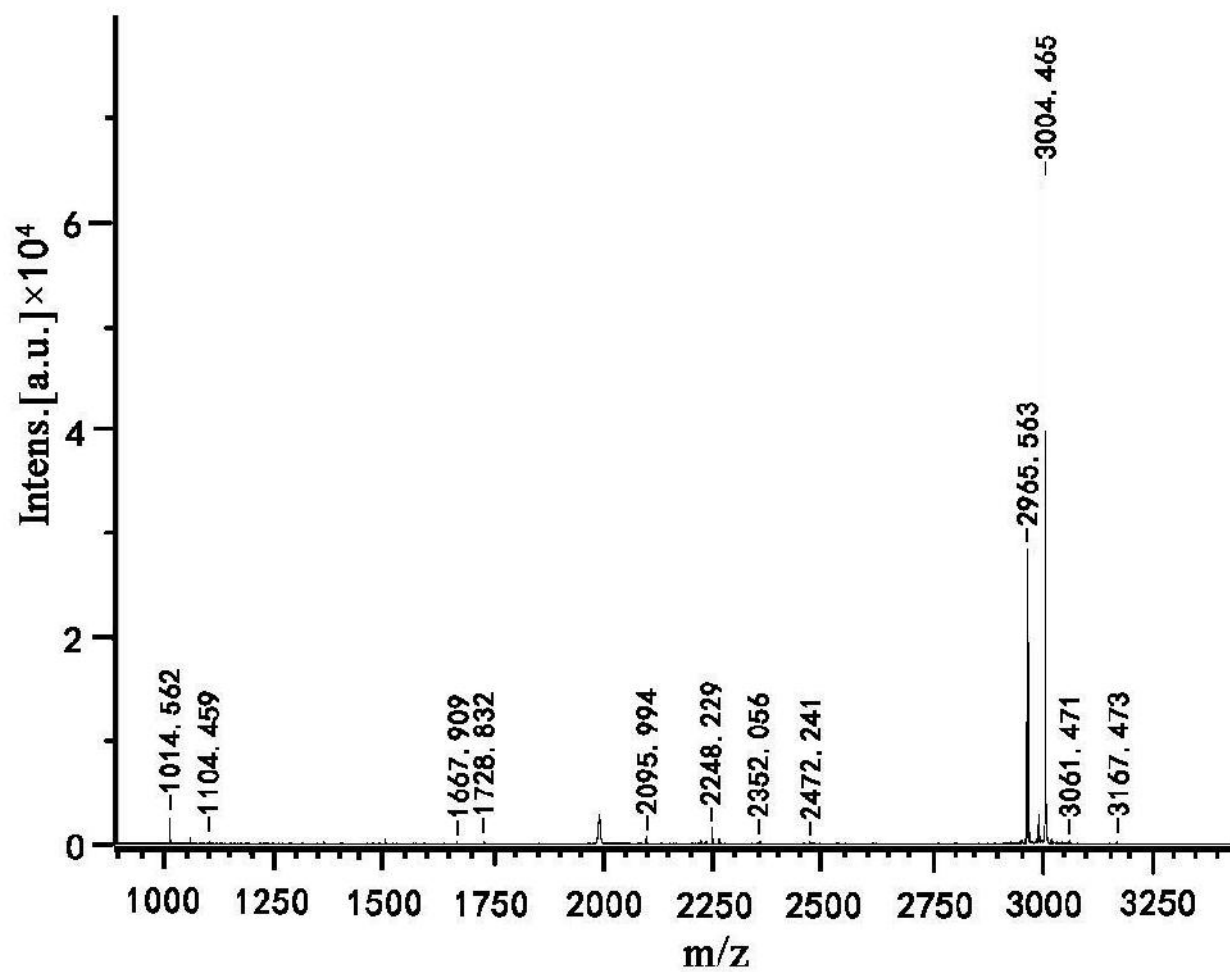


Figure S2

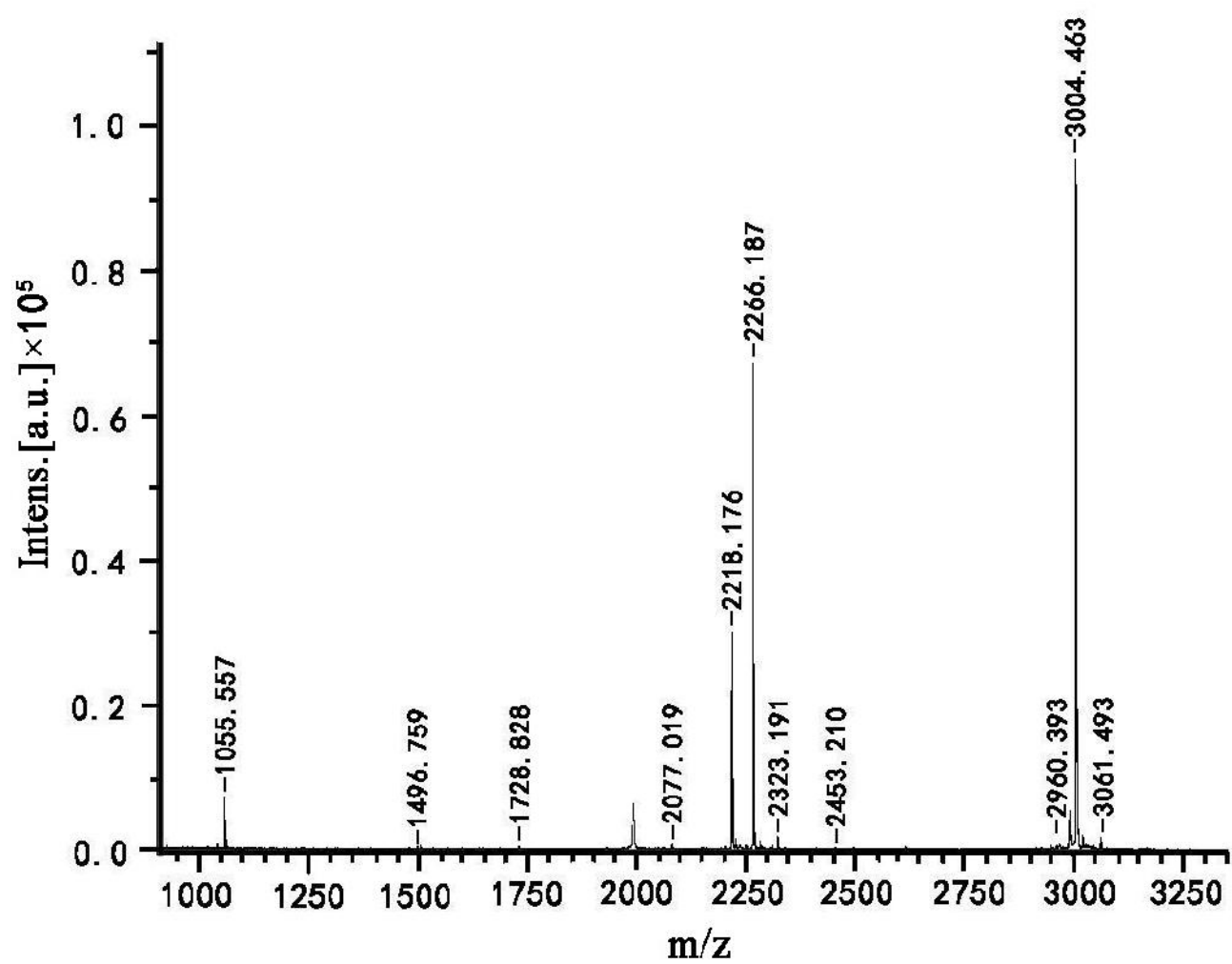


Figure S3

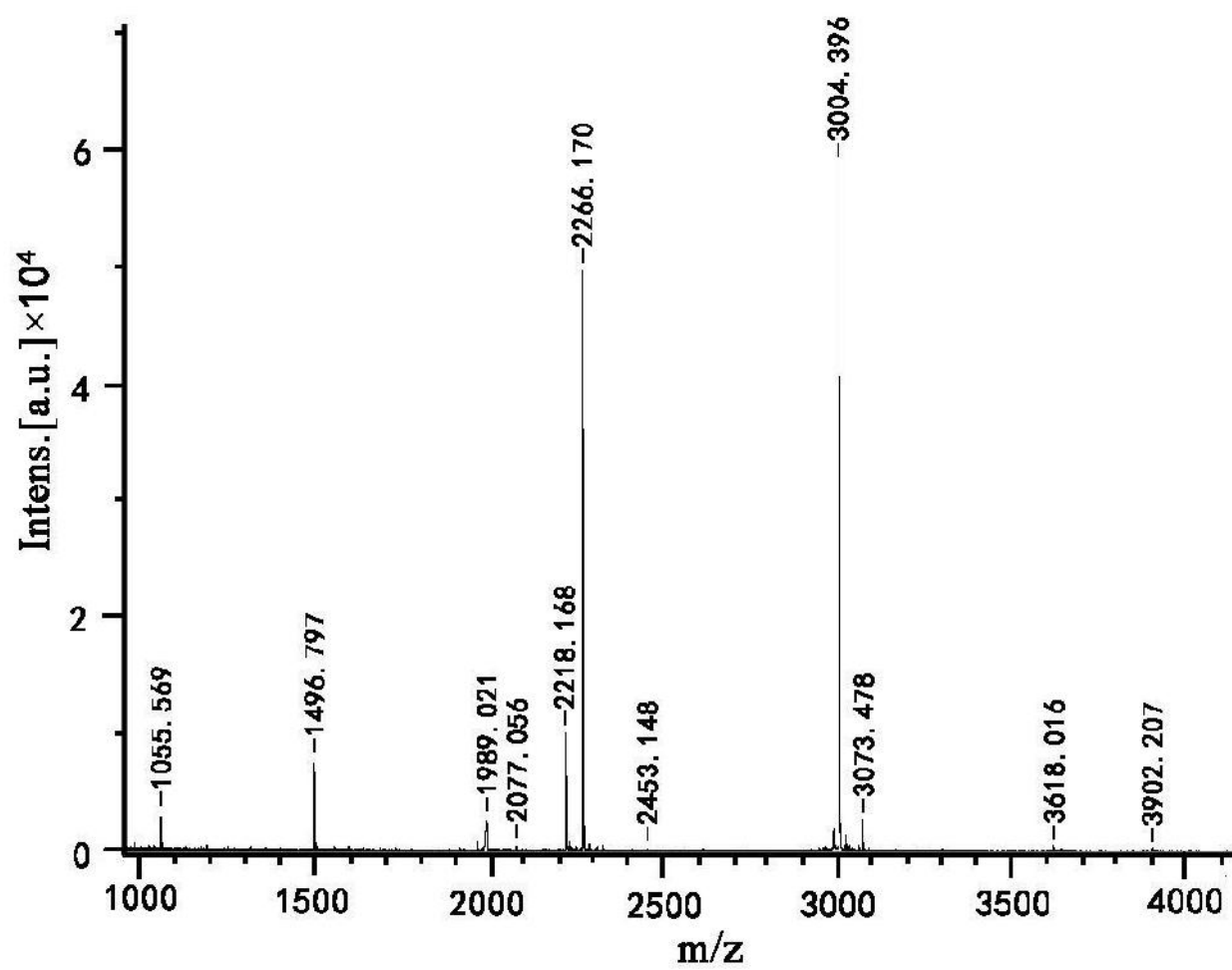


Figure S4