

Glycoproteomic Profile in Wine: A 'Sweet' Molecular Renaissance

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Glycoproteins are believed to be important in several technological, oenological and allergological processes due to their physicochemical properties. The knowledge of the protein glycosylation status in wine will aid in the understanding of these processes. A multiplexed glycopeptide enrichment strategy in combination with tandem mass spectrometry was performed in order to analyze the glycoproteome of white wine. A total of 28 glycoproteins and 44 glycosylation sites were identified. The identified glycoproteins were from grape and yeast origin. In particular, several glycoproteins derived from grape, like invertase and pathogenesis-related (PR) proteins, and from the yeast, were found after the vinification process. Bioinformatic analysis revealed sequence similarity between the identified grape glycoproteins and known plant allergens. This study is an important step forward in order to investigate the implication of glycoproteins in several processes, like protein stabilization and potential allergenic cross-reactivity in wine.

Keywords: wine • N-linked glycoprotein • tandem mass spectrometry • multiplex analysis • hydrazide chemistry • HILIC • titanium dioxide

Introduction

Wine contains varying amounts of different nitrogenous substances, the most important of which are proteins which greatly affect the clarity and stability of the wine.^{1,2} Grapevine (*Vitis vinifera* L.) and related species have recently been subjected to extensive genomic and proteomic studies because of their economic importance worldwide for both fruit and beverage. Wine proteins have grape and yeast origin due to the fermentation process.³ In particular, grape proteins found in wine are mainly pathogenesis-related proteins (PRs) that accumulate in the grape berries during ripening and are expressed as a result of biotic or abiotic stress.⁴ Because PRs are resistant to proteolytic attack and low pH, they are not lost during vinification and can greatly affect the clarity and stability of the wine.^{5,6} Another important class of proteins released during the alcoholic fermentation from the yeast cell wall is mannoproteins which have been proposed to have a high number of positive enological properties such as preventing haze formation in the wines.^{7,8}

Protein glycosylation is the most common post-translational modification (PTM) and the attachment of glycans is known to affect the function of proteins such as stability, activity, pharmacokinetics, targeting, and immunogenicity.⁹ Protein glycosylation regulates several biological processes such as development, growth, or survival and are involved in different physiopathological conditions, for example, cancer, congenital

disorders of glycosylation, infections, and diabetes.^{10,11} In addition, many known allergenic proteins are glycosylated¹² presumably due to foreign glycan motives or longer survival rates for these proteins.

Previously, only very few studies on the characterization of glycoproteins in wine have been reported,^{13,14} mainly targeting individual arabinogalactan proteins and yeast mannoproteins with little attention to N-linked grape glycoproteins due to technical limitations.^{15,16} However, given the implication of glycoproteins into different organoleptic characteristics such as sensorial quality,¹⁷ foamability,¹⁸ and protein stability against proteolytic degradation,^{13,19,20} a thorough study of the glycoprotein content in wine could improve our knowledge of the molecular composition of wine and possibly discover new targets for the investigation of processes important for wine making and quality control of wine.

There are two main types of protein glycosylation: N-linked and O-linked glycosylation. N-linked refers to the glycan structures attached to the polypeptide chain via an amide bond at asparagine (Asn) residues in the consensus sequence Asn-X-Thr/Ser/Cys (where X can be any amino acid except Proline).²¹ O-linked glycans are attached to the oxygen of a hydroxylated amino acid, commonly Ser or Thr, via an ether bond.²² Efficient glycoproteomics strategies have been developed for analyzing N-linked glycosylation. Most of these strategies rely on efficient enrichment of the glycopeptides as they are difficult to detect and characterize in the presence of nonmodified peptides. The most common enrichment methods are hydrophilic interaction chromatography (HILIC),²³ hydrazide chemistry,²⁴ or titanium dioxide (TiO₂) chromatography.²⁵ In particular, hydrazide affinity capture is based on

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the selective reaction between hydrazide resin and the oxidized glycan *cis*-diols.²⁴ In the HILIC chromatography, the glycopeptide fraction is separated from the nonglycosylated one based on the hydrophilicity of these compounds.²³ These two methods can be applied to all glycosylated peptides. The sialic acid-containing glycopeptides were specifically purified using TiO₂ chromatography as shown previously.²⁵

In this study, we are using three complementary enrichment methods for glycosylated peptides in combination with high accuracy mass spectrometry to identify glycosylated proteins and their glycosylation sites in Chardonnay white wine. Using these strategies, 28 glycoproteins including 44 N-linked glycosylation sites of yeast and grape origin were confidently identified. Many of the glycoproteins originating from grapes were found to be homologous to known allergens from various plants and fruits. This study provides new possible targets for investigating oenological and allergological processes, like wine protein haze and allergenic cross-reactivity, of protein glycan determinants.

Materials and Methods

Wine. Glycoprotein analysis was performed on Chardonnay white wine. The wine used in this study was obtained from a winemaker in Turi (Puglia, Italy). The Chardonnay was made from grape berries harvested in August 2008 pressed without skin maceration and the must settled down at 14–16 °C. Alcoholic fermentation was performed at a temperature of 14–16 °C using the yeast *Fermivin* (DSM). After fermentation, the wine was racked and the SO₂ level was adjusted to prevent oxidation processes and malolactic fermentation. After storage for 1 month at 14–16 °C, the wine was bottled. All the analyses were carried out within 1 month from the wine production to avoid any protein loss.

Protein Extraction. Wine sample was filtered through a 0.45 µm membrane (HA Millipore) and concentrated using Millipore Membrane Centrifugal Filter devices with a molecular-weight cutoff of 3 kDa. Retentates (500 µL) were diluted with 8 vol of an ice-cold ethanol solution containing 15% (w/v) trichloroacetic acid (TCA). Proteins were precipitated at 4 °C for 2 h. After centrifugation at 9500g for 10 min at 4 °C, the supernatant was discarded and the pellet was washed with ice-cold ethanol and centrifuged; then, the supernatant was removed and the pellet was lyophilized. The pellet was solubilized in 100 µL of 6 M urea, 2 M thiourea, and 10 mM DTT. After reduction, the proteins were alkylated with 50 mM iodoacetamide for 40 min at room temperature in the dark.

Starting from 15 mL of filtered white wine sample, 500 µg of proteins was obtained. Protein concentration was measured using Qubit Reagent (Invitrogen). Three sample extractions were run in triplicate.

In-Solution Digestion. The protein sample was in-solution digested, in 1:50 enzyme/protein ratio, with trypsin (Promega sequence grade), overnight at room temperature, after diluting the sample to 1 M Urea. The pH was adjusted to pH 3 with TFA, the samples were centrifuged (15000g, 2 min, room temperature (RT)), and the supernatant was passed through an activated, washed Oasis HLB, SPE column (Oasis, Waters). The peptides were eluted with 70% acetonitrile, 0.1% TFA and subsequently lyophilized prior to glycopeptides enrichment.

Glycopeptide Enrichment. 1. Hydrophilic Interaction Chromatography. Glycopeptide enrichment by HILIC microcolumns was performed as previously described.²⁶ Briefly, 150 µg of enzymatic digest was dissolved in 140 µL of 80%

acetonitrile (ACN) containing 0.1% of trifluoroacetic acid (TFA), and loaded onto a HILIC GELoader tip (Eppendorf, Hamburg, Germany) microcolumn packed with ZIC-HILIC media (Sequant, Umeå, Sweden; particle size 10 µm). After sample loading, the column was washed with 150 µL of 80% ACN containing 0.1% TFA. The bound peptides were eluted with 20 µL of 0.1% TFA. An aliquot of the eluates was used for MALDI-TOF MS analysis, while the remaining sample was lyophilized prior to LC-MS/MS analysis. The glycopeptides were redissolved in 40 µL of 50 mM sodium acetate, pH 5.5, and deglycosylated with 0.5 Unit (U) of *N*-glycosylase A (PNGaseA, Roche Diagnostic) and 10 mU of Sialidase A (Glyko) for 16 h at 37 °C. The deglycosylated peptides were analyzed by reverse-phase LC-ESI-MS/MS.

2. Titanium Dioxide Enrichment. TiO₂ enrichment was performed as described previously²⁵ with some modifications. Briefly, peptide mixtures (150 µg) were diluted five times in loading buffer (1 M glycolic acid in 80% acetonitrile, 5% TFA) and incubated with 0.5 mg of TiO₂ beads (5 µm, GL Sciences) for 20 min. After centrifugation, the supernatant was removed and the beads were washed with loading buffer and with 80% ACN in 2% TFA solution. After washing with 20% ACN, 0.2% TFA, bound sialic acid-containing glycopeptides were eluted from TiO₂ beads using 20–40 µL of ammonia–water (10 µL of 25% ammonia solution in 490 µL of water), pH 11. A small aliquot of each of the eluates was acidified with 100% formic acid, purified using a Poros R3 reversed phase microcolumn as described elsewhere,²⁷ and analyzed by MALDI MS. The remaining eluate was lyophilized. The sialic acid-containing glycopeptides were deglycosylated using PNGase A and Sialidase A prior to LC-MS/MS analysis.

3. Hydrazide Chemistry Enrichment. Hydrazide affinity capture was performed as described previously.²⁴ The dried eluate from the C18-SPE desalting step was redissolved in coupling buffer (100 mM sodium acetate, 150 mM NaCl, pH 5.5) and treated with sodium periodate (15 mM final concentration, 30 min, RT) by end-over-end rotation in the dark. The oxidation reaction was quenched with 20 mM sodium sulfite (20 mM final concentration, 15 min, RT) before adding the sample to the 200 µL of hydrazide resin slurry (Affi-Gel Hz, Bio-Rad). The coupling reaction was performed overnight at 37 °C with end-over-end rotation. The resin was washed sequentially twice each with H₂O, 1.5 M NaCl, methanol, acetonitrile, and finally with 50 mM sodium acetate, pH 5.5. The slurry was then treated with PNGase A (New England BioLabs, Inc.) and Sialidase A (Glyko, Novato, CA) overnight at 37 °C. After incubation, the solution was centrifuged (15 000g, 2 min, RT). The supernatant was transferred to another tube and the beads were washed with water/ACN (50/50, 1 mL); then, the liquid was recovered after centrifugation and combined together with the supernatant in the previous Eppendorf tube. The pooled sample was lyophilized.

Deglycosylation Procedure. Glycopeptide-enriched fraction was lyophilized and redissolved in 40 µL of 50 mM sodium acetate, pH 5.5, containing 0.5 U of PNGaseA (PNGaseA, Roche Diagnostics GmbH). To increase the efficiency of PNGaseA (Roche diagnostic), Sialidase A (Glyko, X5006) was added to the incubation buffer as described above.

MALDI-TOF MS Analysis. MALDI-TOF was performed on a Bruker Ultraflex II MALDI-TOF-TOF (BrukerDaltonics, Bremen, Germany). The purified glycopeptides were eluted from the Poros R3 microcolumn directly onto the MALDI-target using 1 µL of DHB matrix solution (20 mg/mL DHB in 70% ACN, and

0.5% formic acid (FA)). Spectra were obtained in positive and negative linear ion mode. Peptide masses were submitted to an analysis program, Flex analysis from Bruker Daltonics (Bremen, Germany).

LC–MS/MS Analysis. The samples were analyzed by a nanoflow HPLC (EasynLC; Proxeon Biosystem, Odense, Denmark) coupled online via a nanoelectrospray ion source (Proxeon Biosystems) to a LTQ–Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were loaded onto a C₁₈-reversed phase column (15 cm long, 100- μ m inner diameter, packed in-house with ReproSil–Pur C₁₈-AQ 3- μ m resin) in buffer A (0.1% FA) with a flow rate of 550 nL/min. The peptides were eluted with a linear gradient from 2% to 40% buffer B (90% ACN and 0.1% FA solution) at a flow rate of 250 nL/min over 50 min. After each sample, the column was washed with 90% buffer B and re-equilibrated with buffer A.

Mass spectra were acquired in the positive ion mode applying a data-dependent automatic switch between survey MS scan and tandem mass spectra (MS/MS) acquisition. Samples were analyzed by acquiring one Orbitrap survey MS scan in the mass range of m/z 300–2000 in the orbitrap, followed by MS/MS of the five most intense ions in the LTQ. The target value in the LTQ–Orbitrap was 1 000 000 ions for survey scan at a resolution of 60 000 at m/z 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Fragmentation in the LTQ was performed by collision-induced dissociation with a target value of 20 000 ions. Ion selection threshold was set to 50 000 counts. Selected sequenced ions were dynamically excluded for 30 s.

Database Search. Peak lists (mgf files) were extracted from the raw data using the program “DTASupercharge” (<http://msquant.sourceforge.net/>). Database searches were performed using the search program MASCOT (Version 2.1.0 and 2.2.04, Matrix Science). The search parameters were set to: MS accuracy 10 ppm, MS/MS accuracy 0.5 Da, Trypsin digestion with two missed cleavage allowed, fixed carbamidomethyl modification of cysteine and variable modification of oxidized methionine, and deamidated asparagine. The tandem mass spectra from the MS/MS experiments were searched against the NCBI nr, nonredundant database, downloaded April 2009. Searches were performed against all species. The searches were performed with a requirement of one unique peptide for protein identification, with an individual Mascot ion score cutoff set for 95% significance. One unique peptide was chosen as minimum requirement because we performed specific enrichment of glycosylated peptides, and therefore, in many cases, we obtained only one deglycosylated peptides due to the site occupancy. Mascot searches of the data set against a decoy database resulted in a false discovery rate of less than 1.3% not identifying any proteins with the applied significance cutoff limits. All identifications were manually validated.

Bioinformatics Analysis. Sequence homology study was performed using Structural Database of Allergenic Proteins (SDAP <http://fermi.utmb.edu/SDAP/>) to identify any allergen presenting an *E*-score less than 0.01. The identified proteins were assigned a Gene Ontology (<http://www.geneontology.org>) term according to their molecular function, and they were statistically grouped into functional categories using Protein Center software (Proxeon Bioinformatics, www.proxeon.com) to identify overrepresented GO categories.

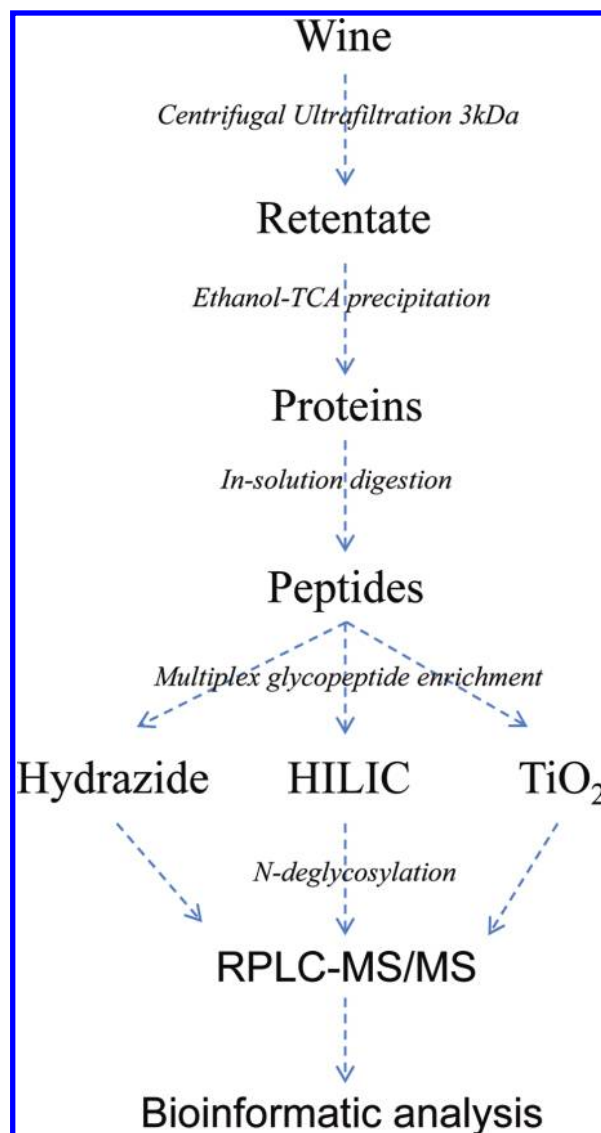


Figure 1. Analytical strategy for the determination of the N-linked glycoproteins present in the wine. Wine sample was concentrated using centrifugal filtration to remove also metabolites and molecules below 3 kDa and retaining the protein fraction. After precipitation using acetone–TCA, the resulting proteins were proteolytically digested using LysC and Trypsin. The obtained peptides were enriched for glycopeptides using three different methods. The glycopeptides were enzymatically deglycosylated and analyzed by reversed phase LC–MS/MS. The identified proteins were subjected to bioinformatic analysis.

Results

Analytical Strategy. In this study, the wine N-linked glycoproteins were characterized using a comprehensive strategy shown in Figure 1. Chardonnay white wine analytes were concentrated by centrifugal ultrafiltration using membrane filters with 3 kDa cutoff prior to ethanol–TCA precipitation. The membrane molecular weight cutoff allowed the concentration of proteins and the removal of ethanol, salts, and small molecules (secondary metabolites and small peptides), whereas the protein precipitation was performed to remove large molecular weight nonproteinaceous compounds. After precipitation, the protein pellet was resolubilized in urea-containing buffer and digested using trypsin after reduction and alkylation. The glycopeptides were enriched from the peptide

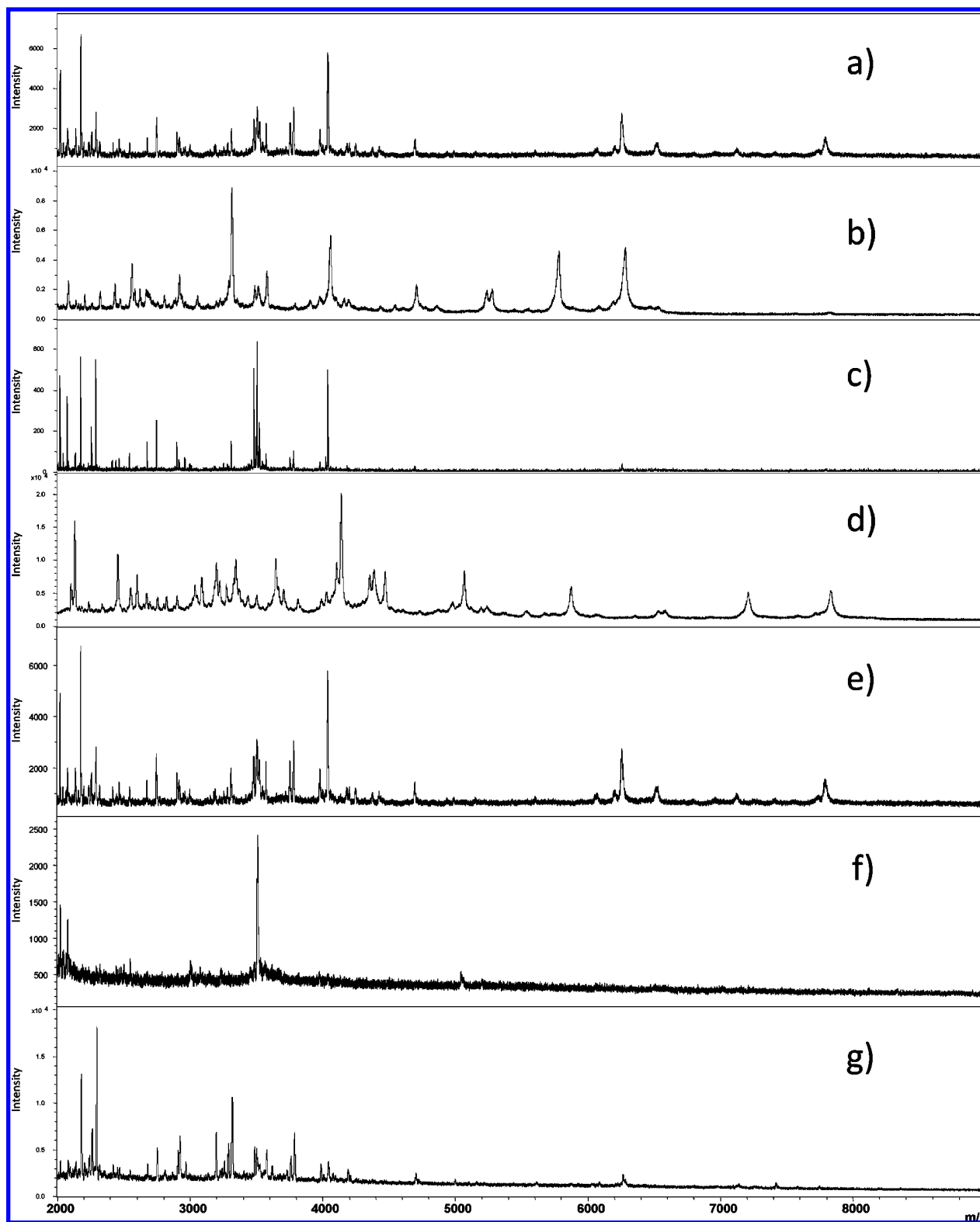


Figure 2. TiO_2 enrichment test. MALDI MS normalized spectra (2000–8000 m/z) acquired on (a) tryptic peptides (linear positive mode), (b) glycopeptides after TiO_2 enrichment (linear positive mode), (c) tryptic peptides (linear negative mode), (d) glycopeptides after TiO_2 (linear negative mode). HILIC enrichment test. MALDI MS normalized spectra (1000–8000 m/z) acquired on (e) tryptic peptides (linear positive mode), (f) flow-through HILIC (linear positive mode), and (g) glycopeptides after HILIC (linear positive mode).

mixture using three independent methods: hydrazide affinity capture, HILIC, and TiO_2 chromatography, taking advantage of different physicochemical characteristics of the glycopeptides. After glycopeptide enrichment, the glycan moiety was enzymatically released using PNGaseA. PNGaseA was used to

cleave the oligosaccharides containing a fucose $\alpha(1-3)$ -linked to the asparagine-linked *N*-acetylglucosamine, commonly found in glycoproteins from plants.²⁸ Since PNGaseA is not active when the glycan structures contain sialic acid, Sialidase A was added. The deamidated peptides were analyzed

by mass spectrometry and the resulting spectra searched using a database search engine (Mascot). Using mass spectrometers with sufficient mass resolution, it is possible to clearly distinguish between asparagine and deamidated asparagine (± 0.984 Da), and directly confirm the physiological sites of N-glycosylation on the identified peptides/proteins, with high confidence. Moreover, the consensus sequence was used to unambiguously localize the glycosylation site. Bioinformatic tools were used to speculate the role of glycosylation.

1. MALDI MS Analysis of Wine Glycopeptides. Before analyzing the glycopeptides by LC–MS/MS, a small fraction was analyzed by MALDI-TOF to evaluate the enrichment efficiency as shown in Figure 2. Tryptic peptides from the wine proteins were acquired in linear positive and negative mode as shown in Figure 2, panels a and c, respectively. As evident from the spectra, many peptide signals are present in the low mass range, below 4000 *m/z*. After TiO₂ enrichment, the MALDI-TOF spectra acquired in linear positive and negative mode are shown in Figure 2, panel b and d, respectively. Here several signals appear in the high mass range clearly indicating an enrichment of the high molecular weight glycopeptides. Moreover, in the spectrum from the negative ion mode, several signals are more intense than in positive mode verifying the purification of acidic sialic acid containing glycopeptides using TiO₂.²⁵ A similar enrichment was performed using HILIC. Tryptic peptides acquired in linear positive mode (Figure 2e) are present in the low mass range, below 4000 *m/z* as shown before. After HILIC enrichment, the MALDI-TOF spectra of the flow-through (Figure 2f) show a depletion of the low molecular weight nonglycosylated peptides and the glycopeptide enrichment in the elution fraction Figure 2g.

2. LC–MSMS of Wine Glycopeptides. Glycopeptides from wine were isolated using the three strategies and the deglycosylated peptides were analyzed by nano-LC–MSMS. Using the current strategy, we have identified 28 glycoproteins mapping 44 N-linked glycosylation sites. As shown in Tables 1 and 2, the glycoproteins belong to grape and yeast, respectively. In particular, 15 unique glycopeptides, mapping 16 N-linked glycosylation sites, were assigned to 13 grape proteins. A total of 15 yeast glycoproteins was found in wine with 26 unique glycopeptides mapping 28 glycosylation sites. The three methods enriched overlapping glycosylated peptides but also method-unique glycopeptides as shown in Figure 3. The annotated spectra are included in Supporting Information (Supplementary annotated spectra HILIC, TiO₂ and Hydrazide).

The MS/MS spectrum in Figure 4a shows the complete y and b ion series of the deglycosylated peptide VQYYKDYC-SQLGVSPGDnLTC of class IV endochitinase, where the n corresponds to the N-linked asparagine converted to aspartic acid by PNGaseA treatment. An example of yeast glycoprotein detected using this strategy is reported in Figure 4b. The MS/MS spectrum shows the complete y and b ion series of the deglycosylated peptide FFYSNnGSQFYIR of glycolipid-anchored surface protein, where the n corresponds to the deglycosylated asparagine after PNGaseA treatment.

Many proteins were identified that were similar to the mRNA sequences present in the *V. vinifera* genome. The unnamed or hypothetical proteins were searched for homologous proteins using BlastP against the NCBI nonredundant protein sequence database (Table 1).

Gene Ontology Analysis. The statistical Gene Ontology analysis of the grape glycoproteins was performed using the *Arabidopsis thaliana* Swiss-Prot data set as reference for

Table 1. *V. vinifera* N-Linked Glycoproteins Identified in Wine

<i>V. vinifera</i>				peptide Mascot score	protein accession	protein description	enrichment method ^b
peptide sequence ^a							
241VQYYKYDYSQQLGVSPGD<N>LTC ²⁶¹			56	gil164699029	class IV Chitinase [<i>Vitis pseudoreticulata</i>]	HILIC/Hydrazide	
6091LFLFN<N>ATGV<N>VTASIK ⁶²⁵			109	gil1839578	vacuolar invertase 1, GIN1 [<i>V. vinifera</i> = grape berries, Sultana, berries, Peptide, 642 aa]	TiO ₂ /Hydrazide	
249DYCSQLGVSPGD<N>LTC ²⁶⁴			21	gil2306813	class IV endochitinase [<i>V. vinifera</i>]	TiO ₂	
1255TTGGCN<N>PCTVFKTDEYCCNSGSC<N>ATDYSR ¹⁵⁵			19	gil7406714	putative thaumatin-like protein [<i>V. vinifera</i>]	TiO ₂	
6LTNSNSVL<N>MTAPANK ¹⁷			32	gil147784619	hypothetical protein [<i>V. vinifera</i>]	Hydrazide	
59NAVENDKDCNLNLYNNPSLLQSLNI<N>VTDALQLPK ⁹³			62	gil147834849	hypothetical protein [<i>V. vinifera</i>]	HILIC	
123YS<N>ESFSQVSESPR ¹³⁷			64	gil157357223	unnamed protein product [<i>V. vinifera</i>]	Hydrazide	
288RPTGPIETTYVFAFED<N>KTPELEK ³²²			60	gil225441373	PREDICTED: hypothetical protein [<i>V. vinifera</i>]	HILIC	
122LAV<N>VSAAR ³⁰			66	gil225445553	PREDICTED: hypothetical protein [<i>V. vinifera</i>]	Hydrazide	
170FP<N>FTTGAHDVAEVTK ¹⁸³			33	gil225445553	PREDICTED: hypothetical protein [<i>V. vinifera</i>]	Hydrazide	
184AAFTAC<N>GTPNISHETEGPADIDLDTAGEHYFICTVGSCHSLGQK ²²⁹			32	gil225445553	PREDICTED: hypothetical protein [<i>V. vinifera</i>]	Hydrazide	
238VATD<N>TTIVEMDPGSFR ²⁴⁹			71	gil225447326	PREDICTED: hypothetical protein [<i>V. vinifera</i>]	Hydrazide	
211FYDEDCGQY<N>GSFDGMK ²²⁷			95	gil225454402	PREDICTED: hypothetical protein [<i>V. vinifera</i>]	Hydrazide	
288NTNLMILG<N>VTMQK ²⁹²			70	gil225459538	PREDICTED: hypothetical protein [<i>V. vinifera</i>]	Hydrazide	
173TDEYCCNSGSC<N>ATTYSEFFK ¹⁹³			100	gil225426795	PREDICTED: hypothetical protein [<i>V. vinifera</i>]	HILIC/TiO ₂ /Hydrazide	

^a Peptide sequence with the N-linked glycosylation site indicated by <N>. ^b Enrichment method that allow the identification of the glycopeptide.

Table 2. Saccharomyces cerevisiae N-Linked Glycoproteins Identified in Wine

Saccharomyces cerevisiae						
peptide sequence ^a	peptide Mascot score	protein accession	SGD name ^b	UniProtK B/ Swiss-Prot	protein description	enrichment method ^c
116 ^V V<N>ETIQDK ¹²⁴	64	gi 297485	YNL160W	P38616	GP38 [S. cerevisiae]	HILIC/TiO ₂ /Hydrazide
220 ^F DSSS<N>FSCNALK ²³³	81	gi 207347722	YBR078W	P38248	YBR078Wp-like protein [S. cerevisiae AWRI1631]	Hydrazide
78 ^V VFDEDEKEYIIGTAL<N>MTFLK ³⁸	52	gi 2499716	pgaX (Aspergillus tubingensis)	Q00293	RecName: Full = Exopolysaccharuronase; Short = ExoPG; AltName: Full = Galacturan 1	Hydrazide
188 ^Y SPQYYHFA<N>SSNVLFDGIDISGYSK ²¹⁴	34	gi 2499716	pgaX (A. tubingensis)	Q00293	RecName: Full = Exopolysaccharuronase; Short = ExoPG; AltName: Full = Galacturan 1	Hydrazide
240 ^F TAEFK<N>LSIPVFFSEYGCNEVTTPR ²⁷¹	46	gi 3730	YMR307W	P22146	glycolipid-anchored surface protein [S. cerevisiae]	HILIC/TiO ₂
35 ^F FYSN<N>GSQFYIR ⁴⁷	72	gi 3730	YMR307W	P22146	glycolipid-anchored surface protein [S. cerevisiae]	TiO ₂ /Hydrazide
91 ^V VAL<N>TTLDHSECMK ¹⁰⁵	65	gi 3730	YMR307W	P22146	glycolipid-anchored surface protein [S. cerevisiae]	Hydrazide
48 ^G VAYQADTA<N>ETSGSTVNDPLANYESCSR ⁷⁶	94	gi 3730	YMR307W	P22146	glycolipid-anchored surface protein [S. cerevisiae]	Hydrazide
240 ^F TAEFK<N>LSIPVFFSEYGCNEVTTPR ²⁷¹	22	gi 3730	YMR307W	P22146	glycolipid-anchored surface protein [S. cerevisiae]	HILIC/TiO ₂
110 ^A MLSGAGMLAAMD<N>RT ¹²⁵	84	gi 437732	YMR008C	P39105	Lysophospholipase	HILIC
69 ^H SENG<N>QSTFK ⁷⁹	35	gi 437732	YMR008C	P39105	Lysophospholipase	Hydrazide
9 ^S GLQVLSVSD<N>GTLK ²⁶	93	gi 4814	YKL096W	P28319	YU1 [S. cerevisiae]	HILIC
54 ^G NYV<N>STFGTPGQR ⁸⁸	75	gi 6320556	YDR349C	Q06325	Putative GPI-anchored aspartic protease	Hydrazide
155 ^G DTTVDYRGEFHGVDIPTDKFH<N>YTL-DWAMDK ¹⁸⁶	26	gi 6321628	YGR189C	P53301	Cell wall protein; putative chitin transglycosidase; Ch1p [S. cerevisiae]	TiO ₂
31 ^S CSCTPGCNSDDKCPG<N>KT ⁵⁰	38	gi 6321844	YHR053C	P07215	Metallothionein [S. cerevisiae]	TiO ₂
87 ^Q FAFYSPGFTV<N>NSR ¹⁰²	79	gi 6322290	YJL171C	P46992	GPI-anchored cell wall protein of unknown function; sequence similarity to YBR162C/TOS1	Hydrazide
52 ^I MNY<N>VTER ²⁰⁸	57	gi 6323648	YMR006C	Q03674	Phospholipase B (lysophospholipase); Plb2p [S. cerevisiae]	HILIC/Hydrazide
112 ^A MLGGAGMIAAMD<N>RT ¹²⁷	99	gi 6323648	YMR006C	Q03674	Phospholipase B (lysophospholipase); Plb2p [S. cerevisiae]	HILIC/TiO ₂
185 ^S VNPGGS<N>LTYTIER ²⁰⁰	99	gi 6323648	YMR006C	Q03674	Phospholipase B (lysophospholipase); Plb2p [S. cerevisiae]	HILIC/TiO ₂
305 ^Y LGT<N>VTNGKPVNK ³¹⁸	41	gi 6323648	YMR006C	Q03674	Phospholipase B (lysophospholipase); Plb2p [S. cerevisiae]	Hydrazide
95 ^L S<N>YTR ¹⁰⁰	20	gi 758281	YBR092C	P24031	constitutive acid phosphatase (PH03) [S. cerevisiae]	Hydrazide
305 ^F QCS<N>DTYVR ³⁹⁵	40	gi 758281	YBR092C	P24031	constitutive acid phosphatase (PH03) [S. cerevisiae]	Hydrazide
146 ^I ISF<N>LSDAETGK ¹⁵⁸	71	gi 82795241	YBR286W	P37302	Vacuolar aminopeptidase Y	Hydrazide
94 ^L A<N>YSTPDYGHPTTR ¹⁰⁷	61	gi 82795241	YBR286W	P37302	Vacuolar aminopeptidase Y	Hydrazide
98 ^L F<N>SSSAL<N>ITELYNVAR ¹¹⁵	29	gi 1151944378	YNL160W	A6ZRS4	glycoprotein [S. cerevisiae YJM789]	Hydrazide
265 ^A AFS<N>LTVGGGFIA<N>NTQLK ²⁸⁵	88	gi 974204	YBR078W	P38248	unnamed protein product [S. cerevisiae]	Hydrazide
295 ^V QTVGGAIEVTG<N>FSTILDSSLK ³¹⁵	59	gi 974204	YBR078W	P38248	unnamed protein product [S. cerevisiae]	Hydrazide

^a Peptide sequence with the N-linked glycosylation site indicated by <N>. ^b Saccharomyces Genome Database (<http://www.yeastgenome.org/>) annotation. ^c Enrichment method that allow the identification of the glycopeptide.

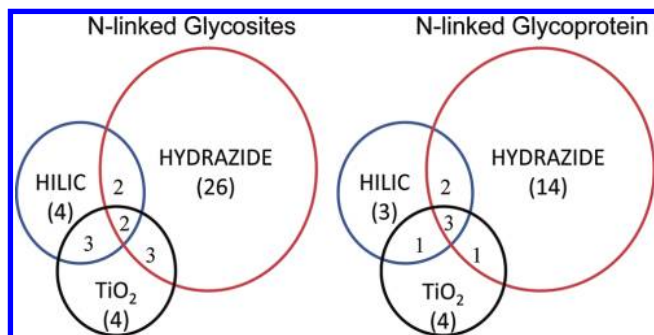


Figure 3. Venn diagram. N-linked glycosites and N-linked glycoprotein overlap between the three glycopeptide enrichment methods (HILIC, TiO₂ and Hydrazide) applied to the characterization of wine glycoproteins.

comparative statistics (Figure 5a,b). The overrepresented biological processes were metabolic processes (47%), response to stimulus (16%), cell organization (16%), and defense response (5%). Moreover, the identified glycoproteins have hydrolase activity (38%), Chitinase activity (13%), and carbohydrate binding activity (12%). The statistical Gene Ontology analysis of the yeast glycoproteins was performed using the *S. cerevisiae* Swiss-Prot data set as reference for comparative statistics (Figure 5c,d). The overrepresented biological processes were metabolic processes (38%), cell organization and biogenesis (16%), response to stimulus (12%), and defense response (4%). Most of the yeast glycoproteins identified are involved in the assembly or disassembly of the cell wall and specifically in the lipid catabolic processes. The overrepresented cellular function for these proteins are hydrolase activity (42%), GPI anchor binding (11%), glycolipid binding activity (11%), and phosphatidylinositol binding (10%).

Discussion

We have shown a strategy for the assessment of glycoproteins from white wine and identified a total of 44 N-linked glycosylation sites in 28 glycoproteins originating from grape and yeast. The small overlap between the three different enrichment methods is presumable due to the different mechanism for glycopeptide enrichment for each method. In particular, hydrazide affinity capture led to the highest number of glycopeptide identifications. As stated above, TiO₂ chromatography is a method specific for sialylated glycopeptides and the small subset of glycoproteins identified using TiO₂ compared to hydrazide affinity capture and HILIC could be explained by this selectivity. Hydrazide chemistry captures all glycopeptides and we therefore expected this method to be the most selective for this study, as clearly illustrated in Figure 3.

Grape Glycoproteins Identified in Wine. The qualitative and quantitative level of protein in wine is related to the chemical environment during wine making. Indeed the physicochemical features of wine such as the low pH, the presence of ethanol, monomeric and polymeric phenolic compounds, and the proteolytic activity of the natural grape and yeast proteases during fermentation allow the presence of proteins that are stable under these conditions.²⁹ Most of these ‘surviving’ proteins have been identified as pathogenesis-related (PR) proteins^{1,30} that are subsequently subjected to several physicochemical changes in bottled wine. Their slow degradation contributes to aggregation and flocculation causing colloidal suspension and subsequently a precipitate. The mechanism of

haze formation is still unknown. Glycosylation has been considered an important factor in protection against protein haze formation in wines.^{20,31} Indeed the presence of glycans on proteins increases stability, solubility, and resistance to proteases.^{32,33}

Here we have identified 13 grape glycoproteins as shown in Table 1. In particular, the grape glycoproteins detected were class IV Chitinase, class IV endochitinase, putative thaumatin-like protein, and vacuolar invertase GIN1. In addition, several unnamed and hypothetical proteins were identified. Indeed, it should be pointed out that in several cases the protein identified by Mascot searches in NCBI database were mRNA sequences present in the *V. vinifera* genome database. This indicates that the annotation of wine proteins is still in its preliminary phase. For most of the identified mRNA sequences, no information is yet available about what proteins they encode and especially which PTMs and which functions they have. In this perspective, this study contributes to the validation of the presence of the protein and the PTM annotation of these proteins. The statistical Gene Ontology analysis of the grape glycoproteins showed that metabolic processes and response to stimulation and defense were overrepresented. Except metabolic processes that compromise a wide definition, the involvement of the glycoproteins in response to stimulus and defense response is related to the predominant presence of pathogenesis-related proteins (PRs) in the wine. Moreover, the identified glycoproteins have hydrolase activity (38%), Chitinase activity (13%), and carbohydrate binding activity (12%) and this is also confirmed by the glycosyl hydrolase activity of the PR proteins which were found to be glycosylated (Chitinase, endochitinase, vacuolar invertase, and hypothetical proteins gil225441373 and gil225454402).

Class IV Chitinase and class IV endochitinase are enzymes that cleave poly- β -1,4-*N*-acetyl-D-glycosamine (chitin) and are classified as PR proteins, in particular belonging to the PR-3 family. Several types (classes) and isoforms of chitinases with different substrate specificities and specific activities³⁴ are constitutively expressed in plants and their expression is enhanced by pathogen infection, and other stress conditions. It has been reported that the level of Chitinase is reduced in grape juices infected with *Botrytis cinerea*.³⁵

Thaumatin-like proteins (TLP) belongs to the group 5 of PR proteins (PR-5) and they are expressed in physiological conditions in many ripe fruits. Moreover, they have been shown to be involved in resistance to pathogen and disease, or defense toward various stress in grape.³⁶ TLP constitute with the chitinases the most represented grape proteins in wine.³⁷ The presence of chitinases and thaumatin-like proteins has been related to the haze formation in wine.^{1,5}

The grape invertases enzymatically convert the sucrose into glucose and fructose and are expressed in the grape berry pericarp during both plant development, growth, and also during biotic (pathogen) and abiotic (environment) conditions.³⁸ Grape vacuolar Invertase 1, GIN1, identified in this study, is more expressed in the grape berry pericarp compared to the isogene GIN2.³⁹ The GIN1 is a glycoprotein with 12 potential N-linked glycosylation sites,¹⁵ and this heterogeneity has been shown in Chardonnay Champagne wine obtained from *B. cinerea* infected grape.⁴⁰

From the BLAST searches, the hypothetical protein gil147834849, gil225441373, gil225426795, gil225447326, and gil225454402 were shown to be homologous, respectively, to the nonspecific lipid-transfer protein (nsLTP), β -1,3-glucanases, osmotin-like

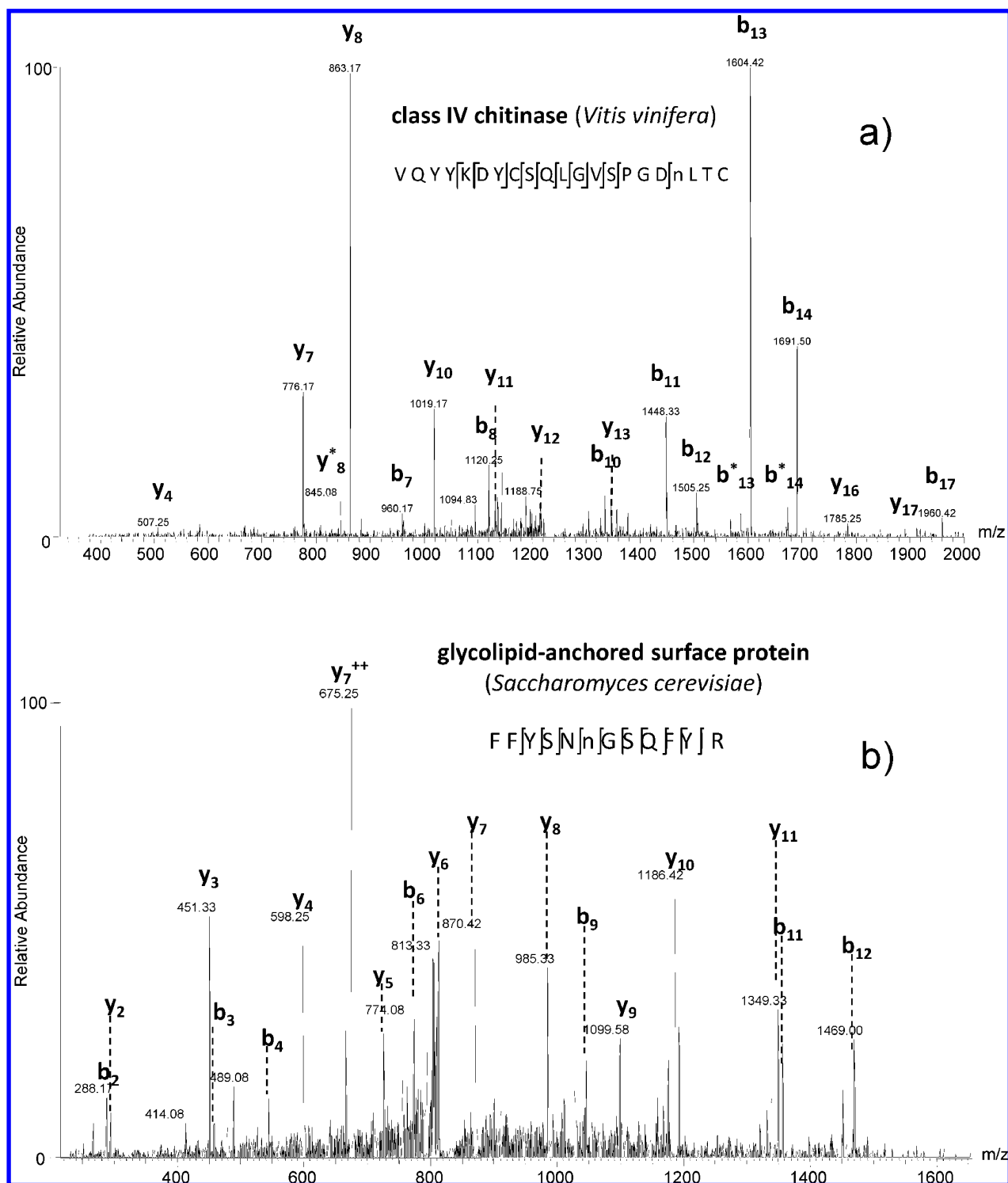


Figure 4. (a) MS/MS of the class IV Chitinase glycosylated peptide VQYYKDYCSQLGVSPGDnLTC. The deamidated asparagine residue within the sequence motif N-X-S/T/C, indicative for the presence of N-linked glycosylation site, is indicated by 'n'. Y and b ions marked with an asterisk (*) indicate the loss of water. (b) MS/MS of the glycolipid-anchored surface (*S. cerevisiae*) glycosylated peptide FFYSnGSQFYIR. The deamidated asparagine residue within the sequence motif N-X-S/T/C, indicative for the presence of N-linked glycosylation site, is indicated by 'n'.

protein, peroxidase a, and class III Chitinase. LTPs belong to PR-14 family which transfer phospholipids between membranes and are involved in antifungal activity. nsLTPs have a broad substrate-binding specificity, are resistant to proteolysis, harsh pH changes, or thermal treatments, and can refold to their native structure upon cooling.⁴¹ The physicochemical properties of LTPs have been described as responsible for the

fungal cell wall damage function.⁴² Vit V1 is a 9 kDa LTP found in grape. It has a high homology to proteins in peach and cherry and they have been identified as the major grape and wine allergens in human.⁴³

Plant β 1,3-glucanases and peroxidases are referred to as PR-2 and PR-9 proteins, respectively. Beta-1,3-glucanases exhibit antifungal activity both *in vitro* and *in planta*, as shown by

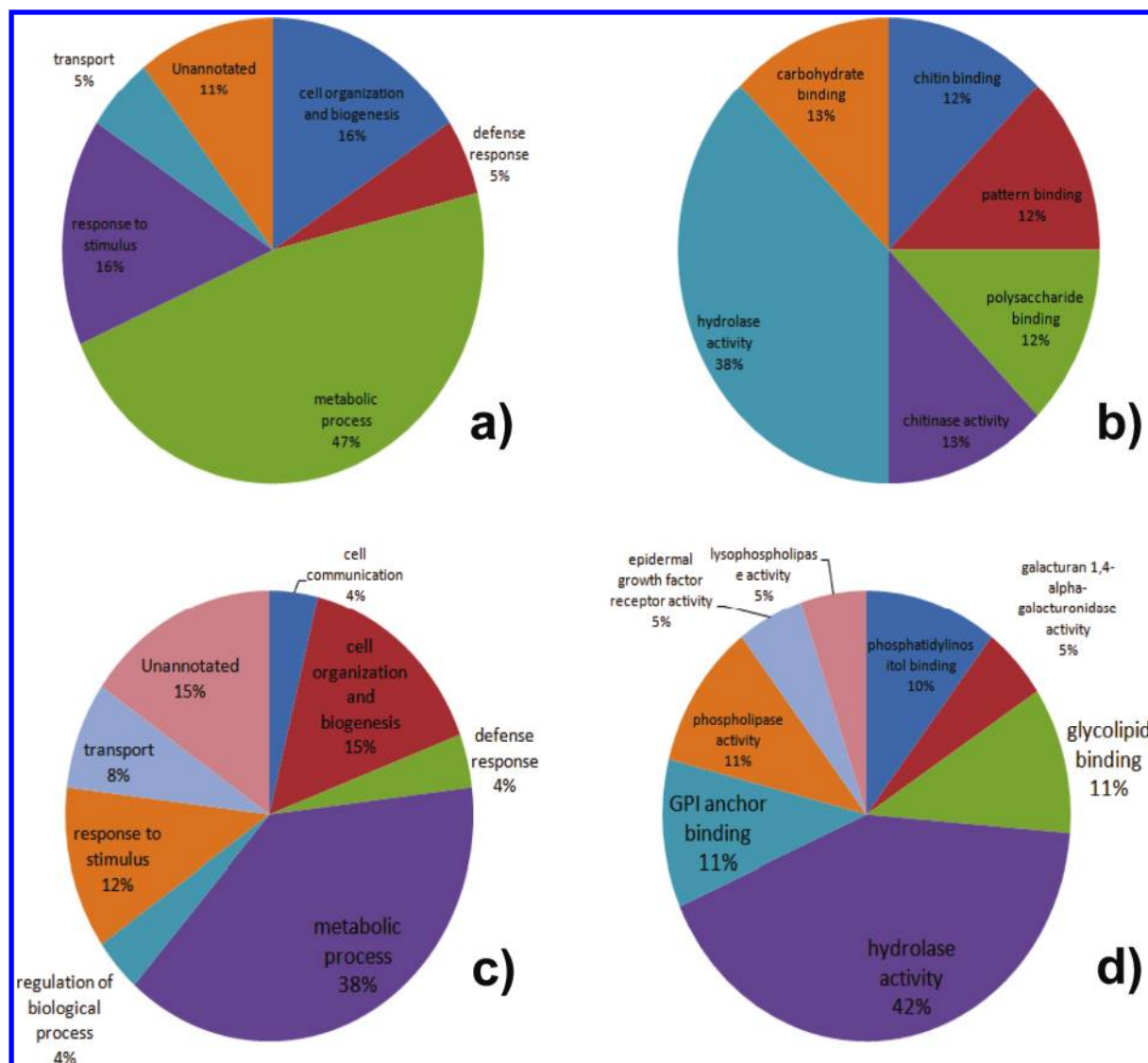


Figure 5. Gene Ontology of a) biological processes and b) molecular functions of the identified grape glycoproteins; c) biological processes and d) molecular functions of the identified yeast glycoproteins.

using transgenic plants overexpressing a PR-2 protein.⁴⁴ A glycosylated peroxidase from wheat flour, Tri a Bd 36K, has been characterized as a glycoprotein allergen.⁴⁵

Yeast Glycoproteins Identified in Wine. Yeast may affect the wine protein composition in two ways: there may be a transfer of yeast proteins to the wine during the process of yeast autolysis and/or the presence of exocellular protease enzymes in the yeasts may contribute to the hydrolysis of the must proteins.⁴⁶ Cell wall derived glycosylated yeast proteins have been found in wines such as mannoproteins.^{14,47}

Using our strategy, we have identified 15 glycoproteins from yeast mapping 28 glycosylation sites as shown in Table 2. The statistical Gene Ontology analysis of the yeast glycoproteins showed similar to the grape glycoproteins an overrepresentation of metabolic processes and response to stimulation and defense. Most of the yeast glycoproteins identified are involved in the assembly or disassembly of the cell wall and specifically in the lipid catabolic processes. The overrepresented cellular function for these proteins is mainly hydrolase activity (42%). The glycoproteins identified are mainly membrane proteins or

covalently attached to the membrane using specific moiety such as a GPI anchor.

Compared to the previous studies that have target specifically the yeast mannoproteins, this strategy allow us to map several glycosylated yeast proteins that extend the knowledge about the released yeast glycoproteins in wine. None of the yeast wine proteins identified here have previously been found in wine.

Structural and Functional Implications of Glycoproteins in Wine. Although technological improvements have allowed the characterization of several glycosylation sites and glycan structures, the biological role of this PTM is not fully understood partly because of the ubiquitous and complex nature of the glycans and the variety of biological processes in which they are involved.⁴⁸ Glycosylation has been reported having a stabilizing effect upon proteins with respect to proteolysis, thermolysis, and other forms of degradation. Indeed, glycosylation stabilizes by steric blockade of cleavage points or alteration of local unfolding kinetics the peptide moiety. Moreover, variation in glycan identity suggests that greater glycan size leads to greater stabilization.^{32,49,50}

Recently, Fusi et al.³¹ have shown that wines with higher concentration of glycoproteins are less susceptible to haze formation. Indeed, after wine glycoprotein-specific precipitation and heat test, they have shown a significant negative correlation between wine stability (haze-forming tendency) and glycoprotein concentration. In addition, it has been shown that yeast mannoproteins prevent haze formation.^{19,20,47,51} The latter findings have resulted in improved wine quality and decreased haze formation overexpressing genes for yeast mannoproteins (Haze protective factors).⁵² On the basis of these findings, it is clear that glycosylation have a significant influence on the wine haze formation and that the list of glycoproteins presented in this study could aid in selecting new target proteins to study this phenomenon in wine or improve the wine making process.

Since most of the grape proteins found in wine are PR proteins, we have chosen to specifically focus on the function and properties of these proteins in relation to their survival in wine. PR proteins are systemically expressed in many plants as a response to stress, pathogenic attack, and wounding^{30,53} but also during fruit development.⁴ Moreover, the fact that many plant food allergens are homologous to proteins of the PR families has been extensively described^{54,55} and these proteins could be important in the development of wine allergy.

The molecular characterization of allergens is a major focus of modern allergology and raises the possibility of more accurate diagnosis and therapy. In the case of plant and insect glycoproteins which bind patients IgE, cross-reactions are very common.^{56,57} This is not unexpected, since the glycan structure is probably the most conserved aspect of these molecules. The presence of β -1,2-xylose and core α -1,3-fucose on N-glycans is more or less ubiquitous throughout the plant kingdom, whereas these moieties are absent from mammals, thus, making them immunogenic. On the other hand, whether cross-reactive carbohydrate epitopes are clinically significant has been a matter of controversy and debate.^{58,59}

The allergenic potential of wine proteins is an aspect that is currently under investigation and is generating great interest. Indeed, during recent years, some case studies of allergic reactions against grapes and wine have been reported.^{60–62} Endochitinase 4A has been described as the major allergen responsible for allergic reactions in wine and a thaumatin-like protein has been described to be a grape allergen.⁴³ In addition, the lipid-transfer protein has been accepted as an allergen by the International Union of Immunological society Allergen Nomenclature Subcommittee.^{43,63} We have here used specific computer algorithms and dedicated databases to screen the amino acid sequence of the identified grape glycoproteins within known allergenic proteins. In particular, we have used the Structural Database of Allergenic Proteins (SDAP <http://fermi.utmb.edu/SDAP/>) to map the hypothetical and unnamed protein sequence against known allergens as shown in Supplementary Table S1. In particular, the hypothetical protein gil147834849 has high homology with Hev b 12 and Pyr c 3, respectively, a latex and pear lipid transfer protein. The hypothetical protein gil225441373 has high homology with Hev b 2 and Ole e 9, respectively, a latex and olive β -1,3-glucanase. The hypothetical protein gil225445553 has high homology with Amb a 3 and Der f 15, respectively, a short ragweed (Ra3) protein and a 98k Chitinase.

These findings show that many of the grape glycoproteins found in this study have homology to known allergens. Future investigations will shed more light on the allergenic potential

of the individual glycoproteins found in this study in order to aid in the development of low allergenic wine.

Conclusions

An investigation of the wine glycoproteome has been addressed in this study using a multiplexed glycoproteomics approach. In this strategy, the physicochemical properties like hydrophilicity, charge, and reactivity of the *cis*-diols of the glycans were used to enrich the wine glycoproteins at the peptide level using a chromatography-based (HILIC and TiO₂) and a chemical-based (hydrazide affinity capture) approach. After the enrichment of the glycopeptides, mass spectrometry was used to characterize the glycosylation sites. Using this strategy, 28 glycoproteins, mapping 44 N-linked glycosylation sites, were confidently identified. The glycoproteins found in the wine are from grape and yeast origin. Bioinformatic analysis of the identified glycoproteins shed new insight on the implication of the glycan moieties in several mechanisms like protein stability and allergenic cross-reactivity. Clearly, the allergenic potential of many of the identified grape glycoproteins is related to the homology with proteins that have been proven to be allergens, and for most of the identified glycoproteins, the word allergen is used due to this primary sequence homology. Further investigations are necessary to investigate the potential for several of the identified glycoproteins to prevent haze formation and the allergenic potential of several of the PR-related proteins in order to validate the importance of glycoproteins in wine.

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Supporting Information Available: Bioinformatic analysis of the N-linked glycoproteins identified in the wine; annotated spectra HILIC, TiO₂, and Hydrazide; This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (63) Sbornik, M.; Rakoski, J.; Mempel, M.; Ollert, M.; Ring, J. IgE-mediated type-I-allergy against red wine and grapes. *Allergy* **2007**, *62* (11), 1339–1340.

PR100298J

Supplementary Table S1:

Bionformatic analysis of the N-linked glycoproteins identified in the wine.

Protein name	Accession number	^a Glycosylation site localization	^b BlastP protein [E-value]	^c Allergen SDAP similarities
class IV chitinase [Vitis pseudoreticulata]	gil164699029	X	-	Mus a 2.0101 [4.8e-18] Pers a 1 [1.3e-17]
vacuolar invertase 1, GIN1 [Vitis vinifera=grape berries, Sultana, berries, Peptide, 642 aa]	gil1839578		-	Lyc e 2.0102 [1.6e-180] Lyc e 2.0101 [4.5e-155]
putative thaumatin-like protein [Vitis vinifera]	gil7406714	X	-	Mus a 4.0101 [7e-65] Act c 2 [5.3e-62]
class IV endochitinase [Vitis vinifera]	gil2306813	X	-	Mus a 2.0101 [1.8e-17] Hev b 11.0101 [3.1e-17]
hypothetical protein [Vitis vinifera]	gil147784619		Putative uncharacterized protein [3e-69] A5AUB3	Sal k 1.0301 [4e-03] Sal k 1.0302 [4e-03]
hypothetical protein [Vitis vinifera]	gil147834849	X	Non-specific lipid-transfer protein[1e-65] A7QVC2	Hev b 12 [8.9e-03]
unnamed protein product [Vitis vinifera]	gil157357223		Chromosome undetermined scaffold_165, whole genome shotgun sequence [0] A7QT18	Sal k 2.0101 [1.7e-24]

PREDICTED: hypothetical protein [<i>Vitis vinifera</i>]	gil225441373	X	Beta-1,3-glucanase [1e-150] B2ZP01	Hev b 2 [8.6e-54] Ole e 9 [2.0e-29]
PREDICTED: hypothetical protein [<i>Vitis vinifera</i>]	gil225445553	X	Dycianin [2e-76] Q9M510	Amb a 3 [9.8e-07] Der f 15 [1.4e-03]
PREDICTED: hypothetical protein [<i>Vitis vinifera</i>]	gil225447326	X	Peroxidase a [3e-133] PODa	-
PREDICTED: hypothetical protein [<i>Vitis vinifera</i>]	gil225454402	X	Class III chitinase [1 e-68] ChiFIII	Ziz m 1.0101 [7.9e-36] Tyr p 13 [7.2e-03]
PREDICTED: hypothetical protein [<i>Vitis vinifera</i>]	gil225459538		Peptidoglycan-binding LysM domain-containing related protein [1e-180] B2ZAQ1	-
PREDICTED: hypothetical protein [<i>Vitis vinifera</i>]	gil225426795	X	Osmotin-like protein [1e-133] OSM1	Act c 2 [4.9e-68] Mus a 4.0101 [4.8e-67]

^a Glycosylation site localized within the PFAM domain (X).

^b BLASTP (protein-protein blast <http://blast.ncbi.nlm.nih.gov/>) search to identify protein sequences homolog to the hypothetical *Vitis vinifera* N-linked glycoproteins.

^c SDAP database (<http://fermi.utmb.edu/SDAP/>) to identify any allergen similar to the N-linked glycoproteins identified presenting a sequence homology with an E score less than 0,01.

Annotated spectra HILIC

MS/MS Fragmentation of **MNYnVTER**

Found in **gi|6323648**, Phospholipase B (lysophospholipase) involved in phospholipid metabolism; displays transacylase activity in vitro; overproduction confers resistance to lysophosphatidylcholine; Plb2p [*Saccharomyces cerevisiae*]

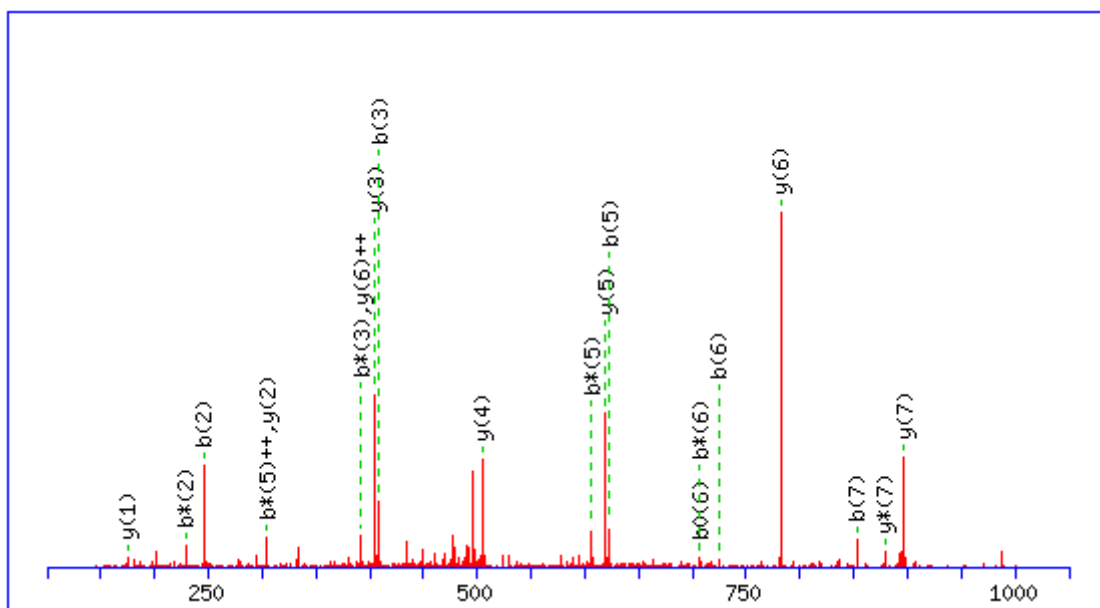
Monoisotopic mass of neutral peptide Mr(calc): 1026.444016

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N4 : Deamidated (NQ)

Ions Score: 57 **Expect:** 0.014



MS/MS Fragmentation of **AMLGGAGMIAAMDnRT**

Found in **gi|6323648**, Phospholipase B (lysophospholipase) involved in phospholipid metabolism; displays transacylase activity in vitro; overproduction confers resistance to lysophosphatidylcholine; Plb2p [*Saccharomyces cerevisiae*]

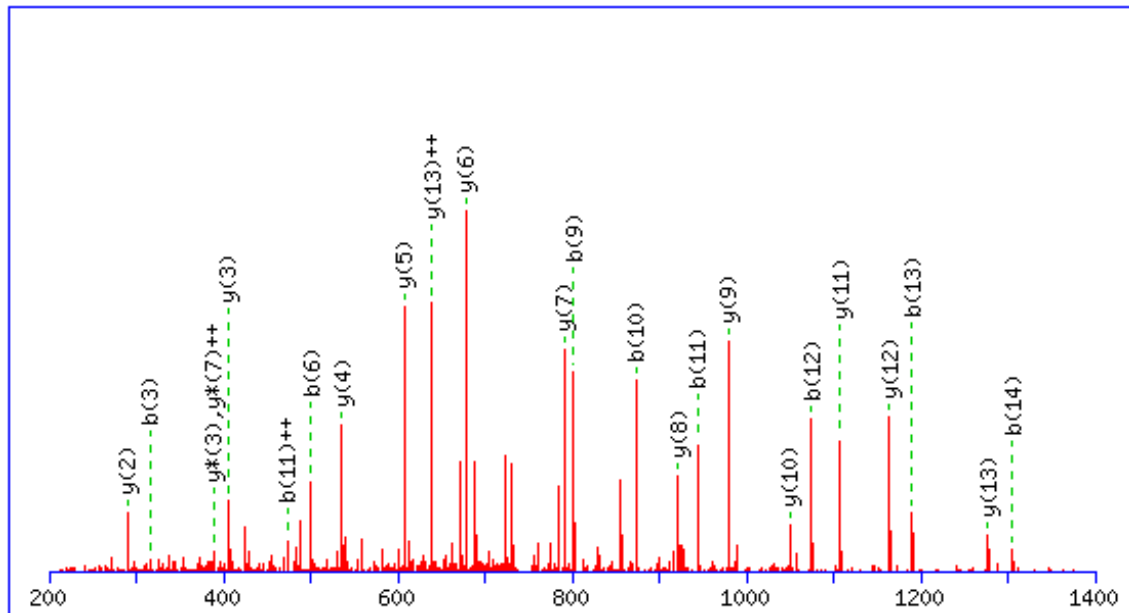
Monoisotopic mass of neutral peptide Mr(calc): 1478.667953

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N14 : Deamidated (NQ)

Ions Score: 79 **Expect:** 0.00014



MS/MS Fragmentation of **SIVNPGGSnLTYTIER**

Found in [gi|6323648](#), Phospholipase B (lysophospholipase) involved in phospholipid metabolism; displays transacylase activity in vitro; overproduction confers resistance to lysophosphatidylcholine; Plb2p [*Saccharomyces cerevisiae*]

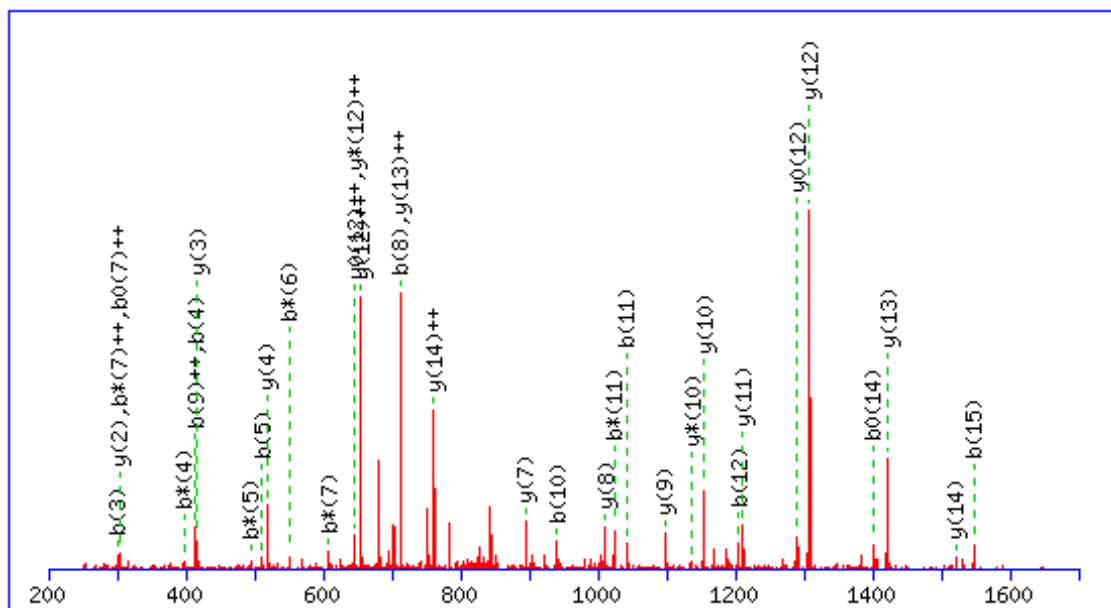
Monoisotopic mass of neutral peptide Mr(calc): 1720.863159

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N9 : Deamidated (NQ)

Ions Score: 99 **Expect:** 1.3e-06



MS/MS Fragmentation of **NAVENDKDCLCNLYNNPSSLQSLN****VTDALQLPK**

Found in **gi|147834849**, hypothetical protein [Vitis vinifera]

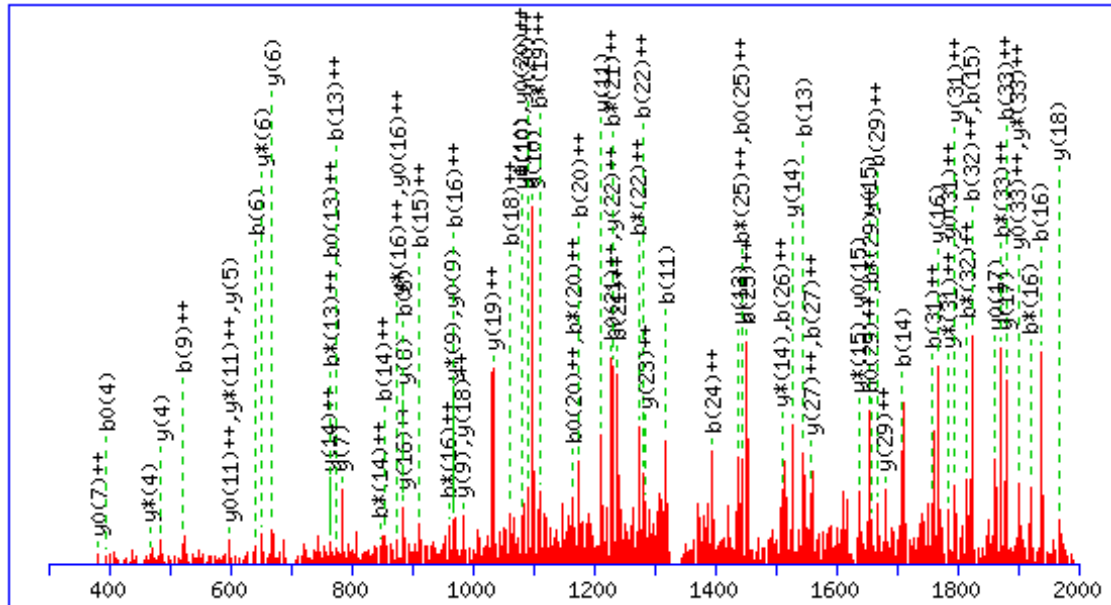
Monoisotopic mass of neutral peptide Mr(calc): 4000.950974

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N26 : Deamidated (NQ)

Ions Score: 62 **Expect:** 0.013



MS/MS Fragmentation of **VVnETIQDK**

Found in **gi|297485**, GP38 [Saccharomyces cerevisiae]

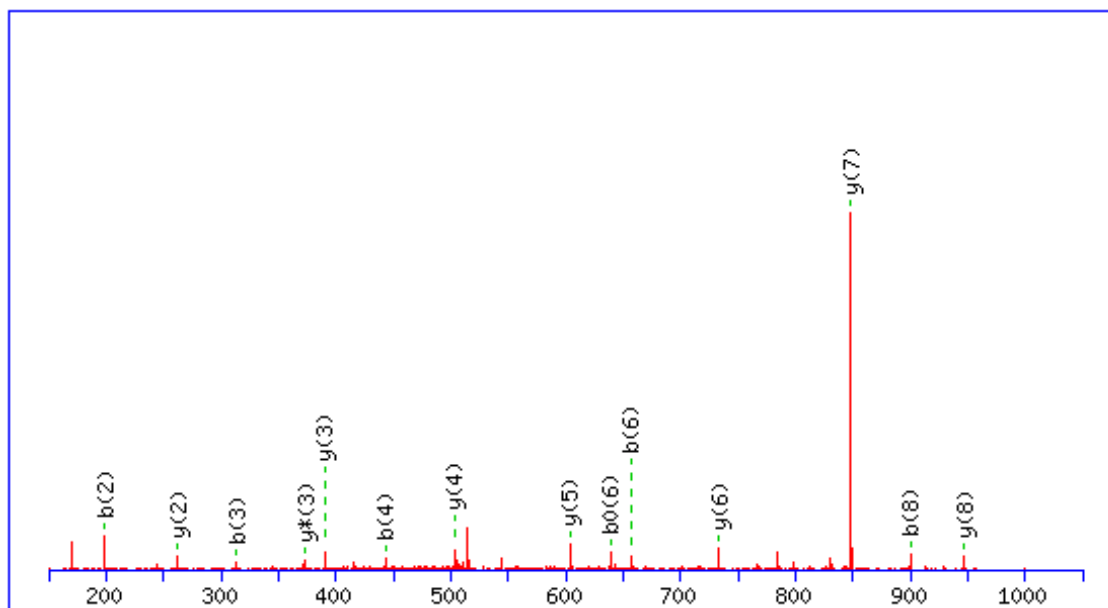
Monoisotopic mass of neutral peptide Mr(calc): 1045.529144

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N3 : Deamidated (NQ)

Ions Score: 62 **Expect:** 0.0042



MS/MS Fragmentation of **AMLSGAGMLAAMDnRT**

Found in **gi|437732**, lysophospholipase

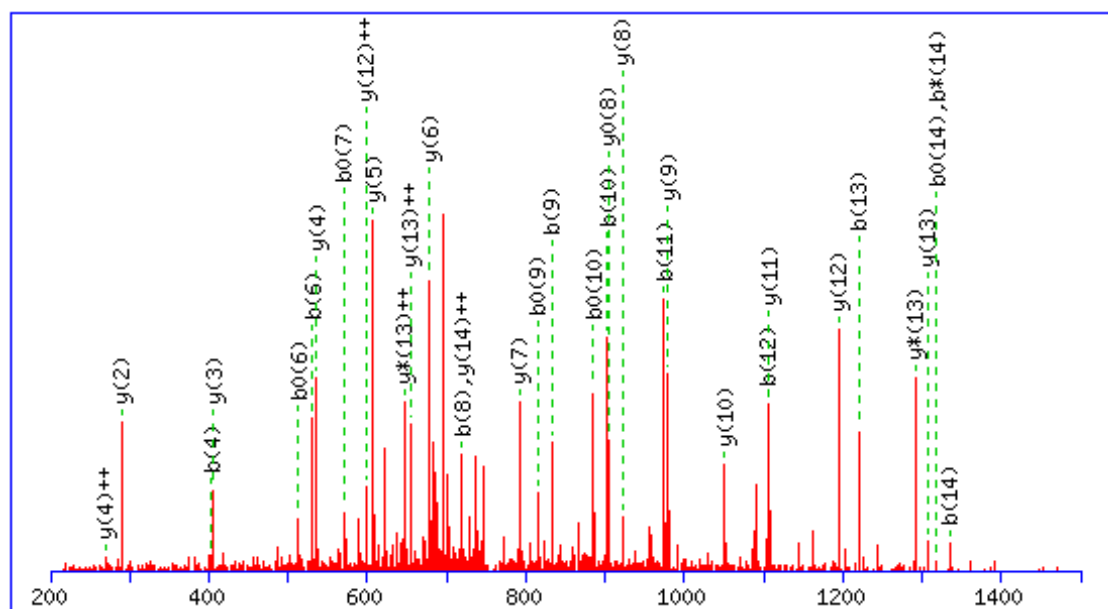
Monoisotopic mass of neutral peptide Mr(calc): 1508.678513

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N14 : Deamidated (NQ)

Ions Score: 84 **Expect:** 4.1e-05



MS/MS Fragmentation of **TAEFKnLSIPVFFSEYGCNEVTPR**

Found in **gi|3730**, glycolipid-anchored surface protein [*Saccharomyces cerevisiae*]

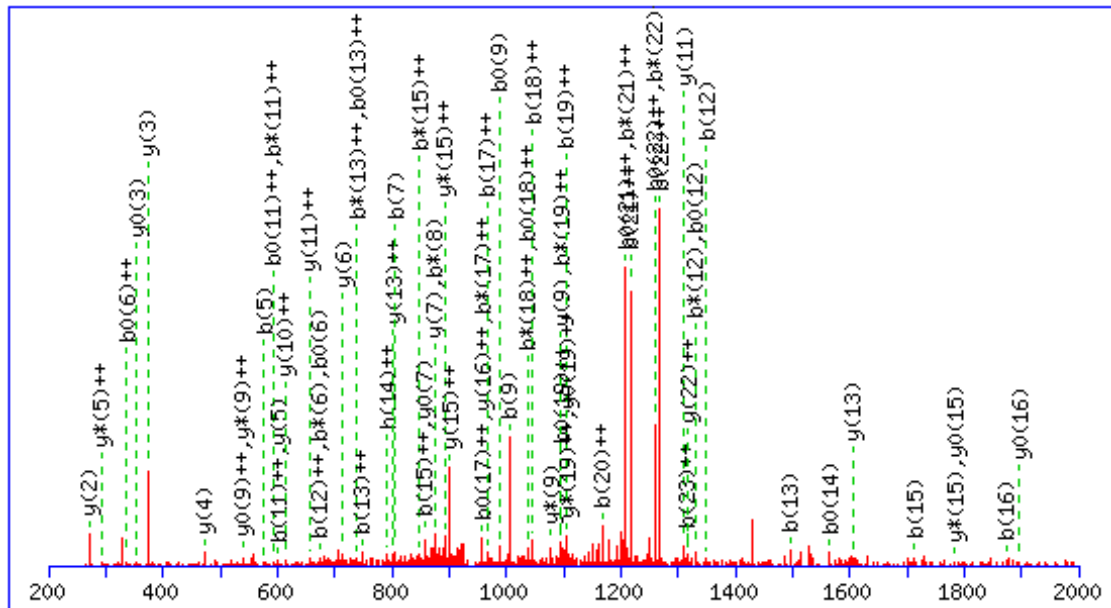
Monoisotopic mass of neutral peptide Mr(calc): 2805.331970

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

Ions Score: 46 **Expect:** 0.59



MS/MS Fragmentation of **TDEYCCNSGSCnATTYSEFFK**

Found in **gi|225426795**, PREDICTED: hypothetical protein [*Vitis vinifera*]

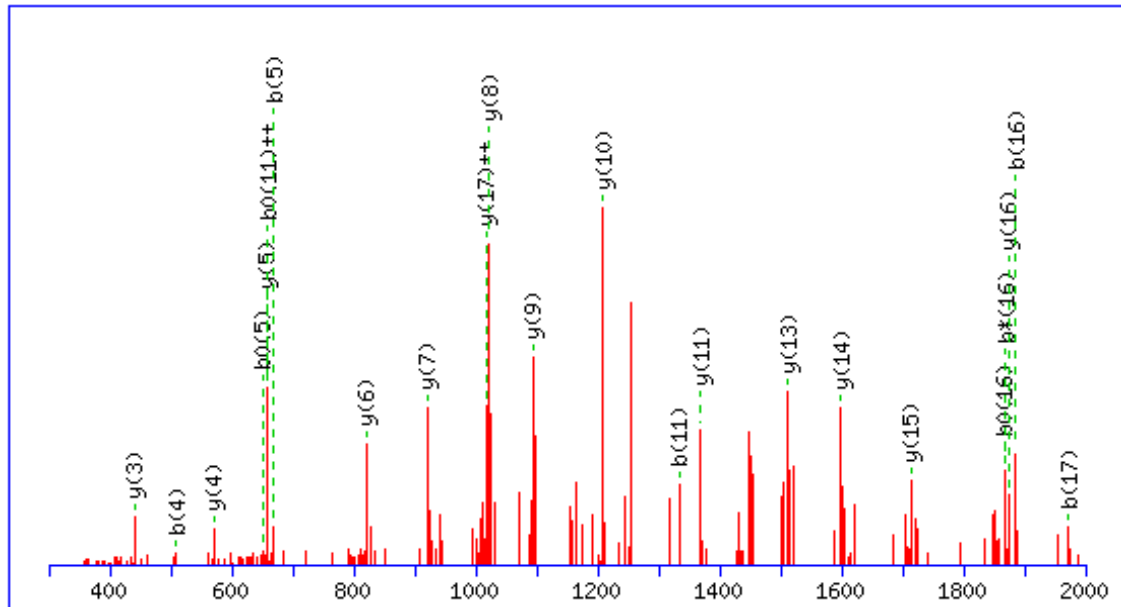
Monoisotopic mass of neutral peptide Mr(calc): 2540.940659

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N12 : Deamidated (NQ)

Ions Score: 100 **Expect:** 1.6e-06



MS/MS Fragmentation of **VQYYKDYCSQLGVSPGDnLTC**

Found in **gi|164699029**, class IV chitinase [Vitis pseudoreticulata]

Monoisotopic mass of neutral peptide Mr(calc): 2468.051193

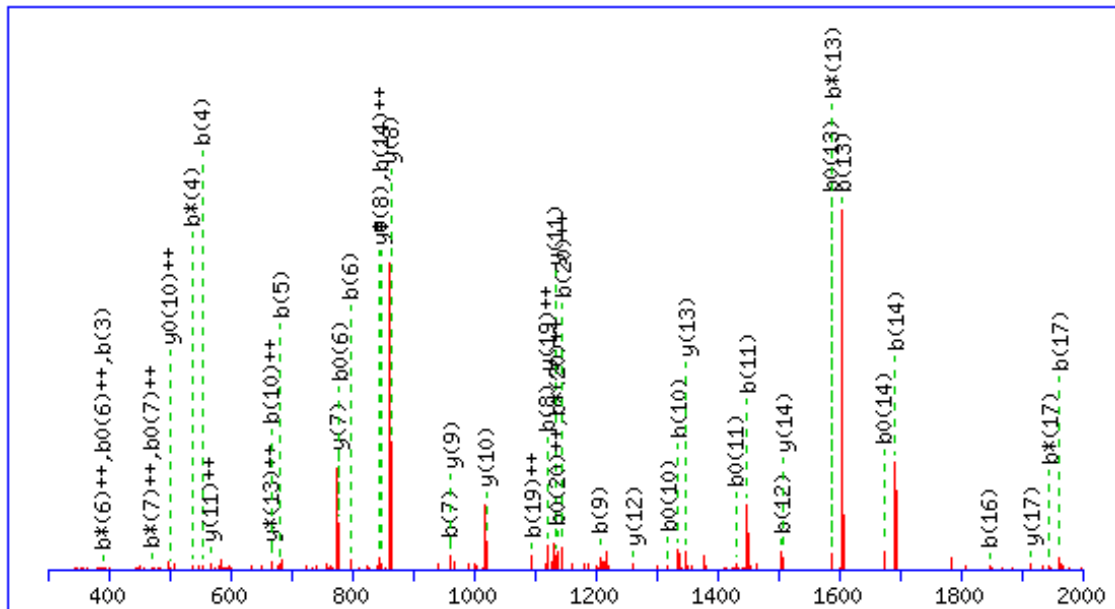
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

Q10 : Deamidated (NQ)

N18 : Deamidated (NQ)

Ions Score: 52 Expect: 0.2



MS/MS Fragmentation of **RPTGPIETYVFMFDEdNKTPELEK**

Found in **gi|225441373**, PREDICTED: hypothetical protein [Vitis vinifera]

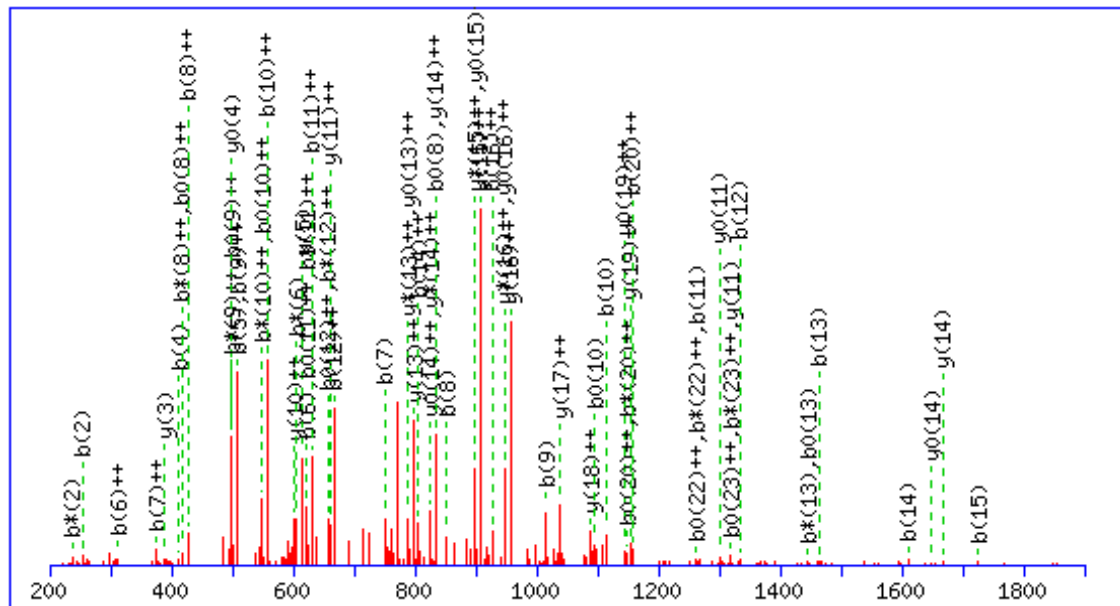
Monoisotopic mass of neutral peptide Mr(calc): 2927.389862

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N18 : Deamidated (NQ)

Ions Score: 79 **Expect:** 0.00054



MS/MS Fragmentation of **SGSDLQYLSVYSDnGTLK**

Found in **gi|4814**, YJU1 [Saccharomyces cerevisiae]

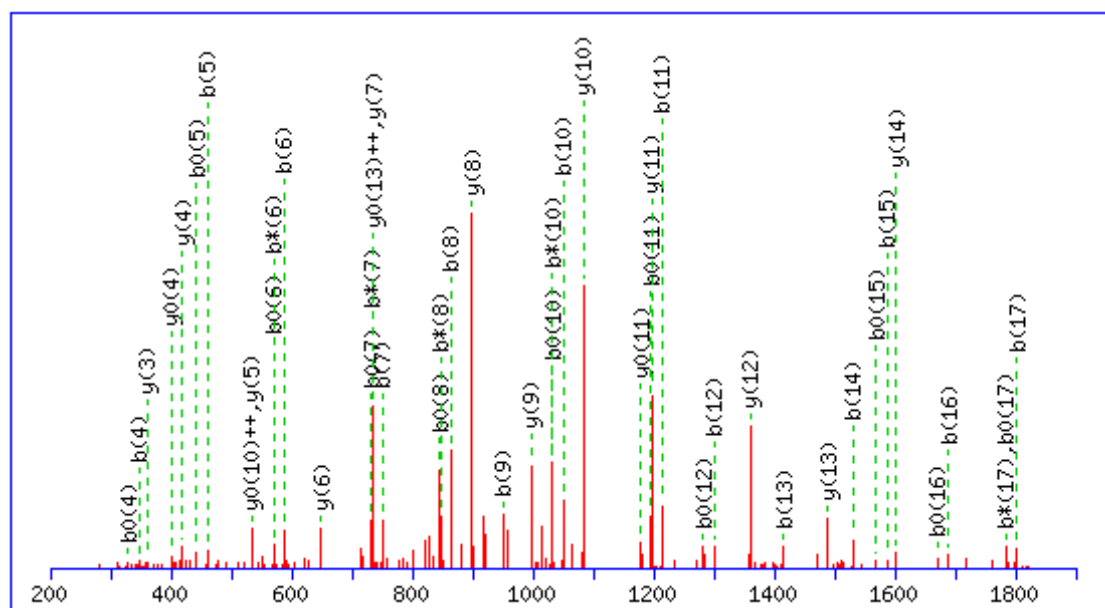
Monoisotopic mass of neutral peptide Mr(calc): 1946.910904

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N14 : Deamidated (NQ)

Ions Score: 109 **Expect:** 3.6e-07



MS/MS Fragmentation of **LFFHDCFVnGCDASVLLDDTSSFTGEQTAVPNK**

Found in **gi|225434381**, PREDICTED: hypothetical protein [Vitis vinifera]

Monoisotopic mass of neutral peptide Mr(calc): 3692.628738

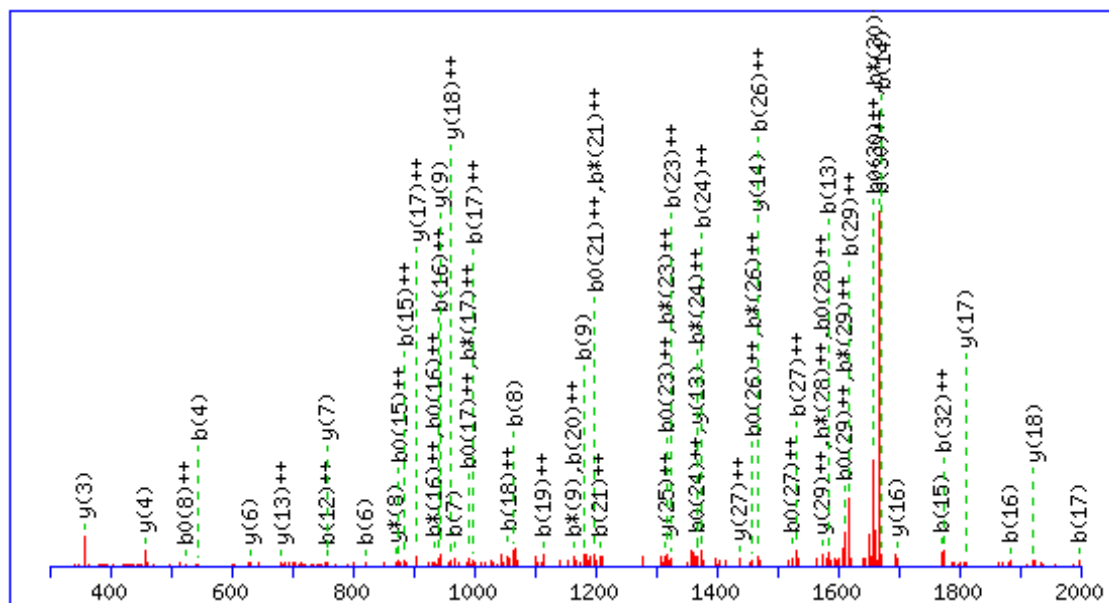
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N9 : Deamidated (NQ)

Q27 : Deamidated (NQ)

Ions Score: 74 **Expect:** 0.0021



Annotated spectra Hydrazide

MS/MS Fragmentation of **LFLFNnATGVnVTASIK**

Found in **gi|157351694**, unnamed protein product [Vitis vinifera]

Monoisotopic mass of neutral peptide Mr(calc): 1809.951248

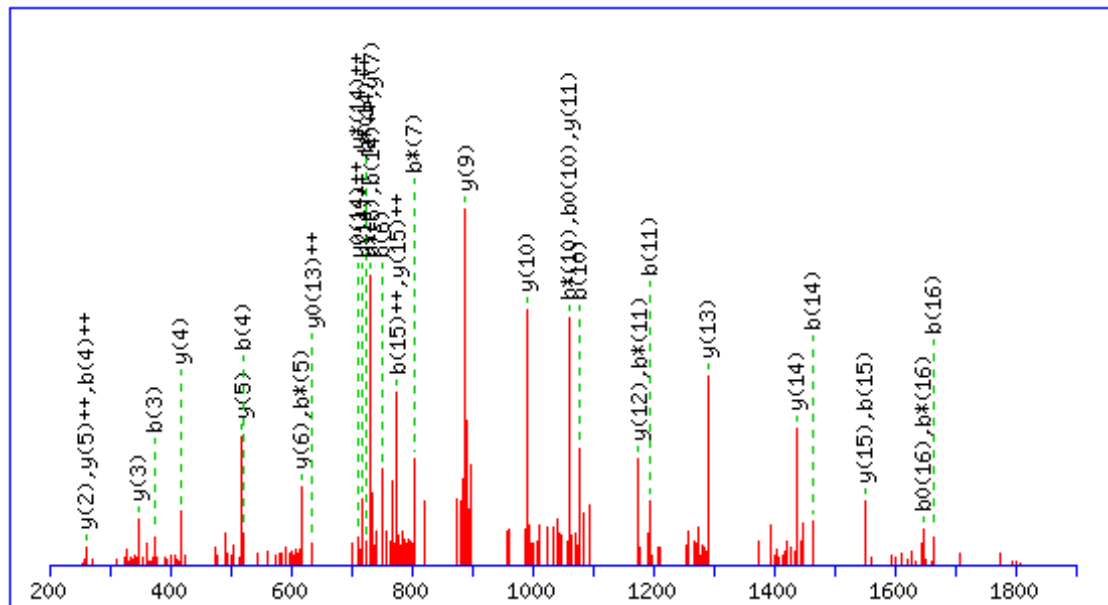
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

N11 : Deamidated (NQ)

Ions Score: 141 **Expect:** 7.7e-11



MS/MS Fragmentation of **AAFSnLTTVGGGFIIAnNTQLK**

Found in **gi|974204**, unnamed protein product [Saccharomyces cerevisiae]

Monoisotopic mass of neutral peptide Mr(calc): 2238.153198

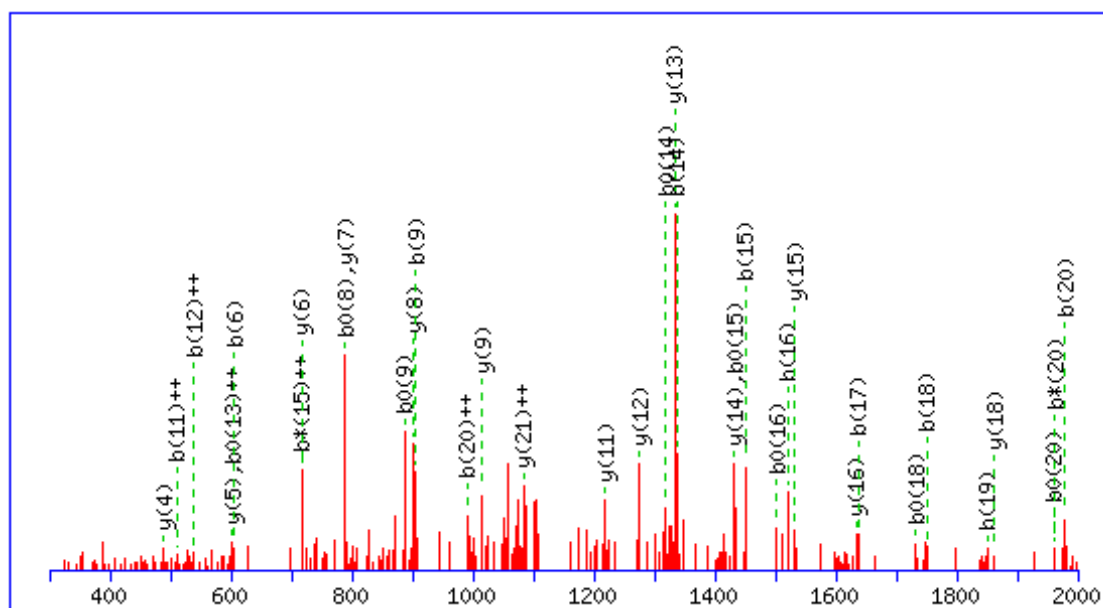
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N5 : Deamidated (NQ)

N17 : Deamidated (NQ)

Ions Score: 97 **Expect:** 2.9e-06



MS/MS Fragmentation of **VQTVGGAIEVTGnFSTLDLSSLK**

Found in **gi|974204**, unnamed protein product [*Saccharomyces cerevisiae*]

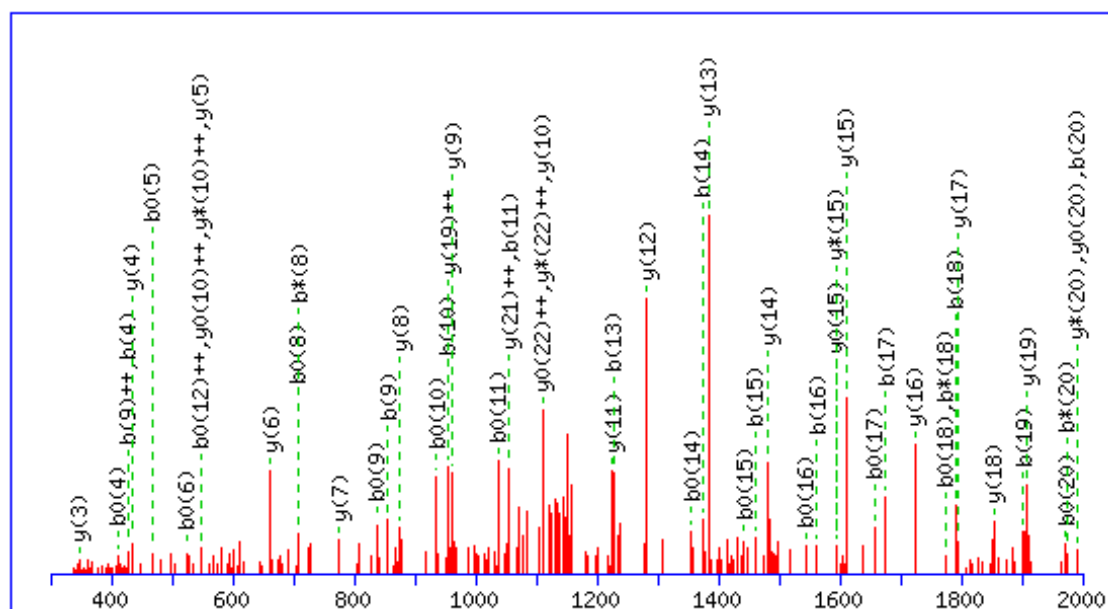
Monoisotopic mass of neutral peptide Mr(calc): 2336.211136

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N13 : Deamidated (NQ)

Ions Score: 150 **Expect:** 1.3e-11



MS/MS Fragmentation of **TDEYCCNSGScnATTYSEFFK**

Found in **gi|225426795**, PREDICTED: hypothetical protein [Vitis vinifera]

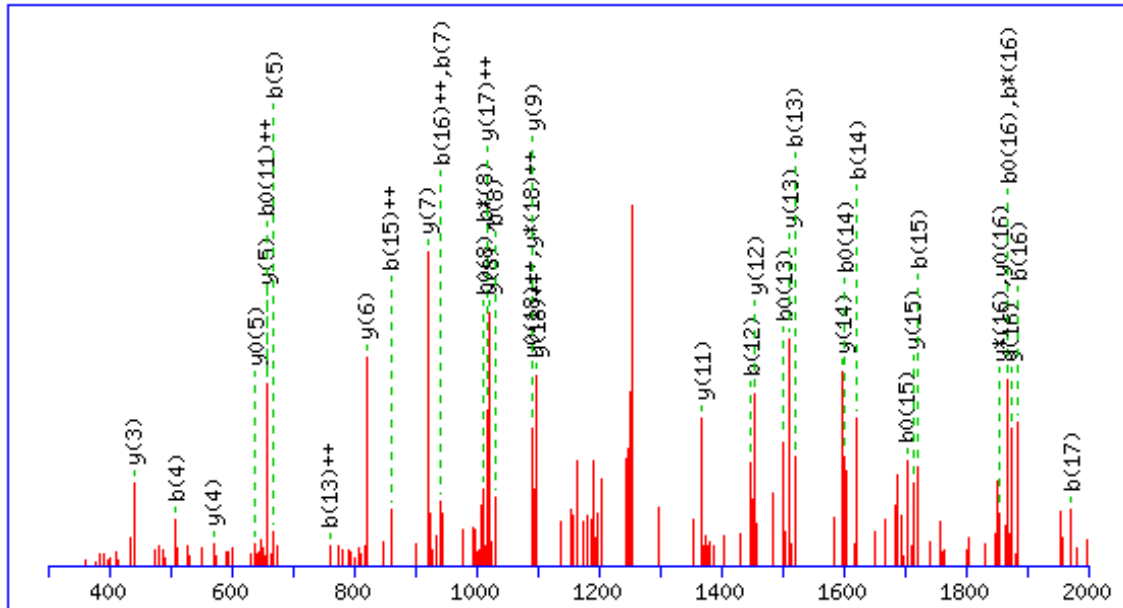
Monoisotopic mass of neutral peptide Mr(calc): 2540.940659

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N12 : Deamidated (NQ)

Ions Score: 75 **Expect:** 0.0002



MS/MS Fragmentation of **VVnETIQDK**

Found in **gi|151944378**, glycoprotein [Saccharomyces cerevisiae YJM789]

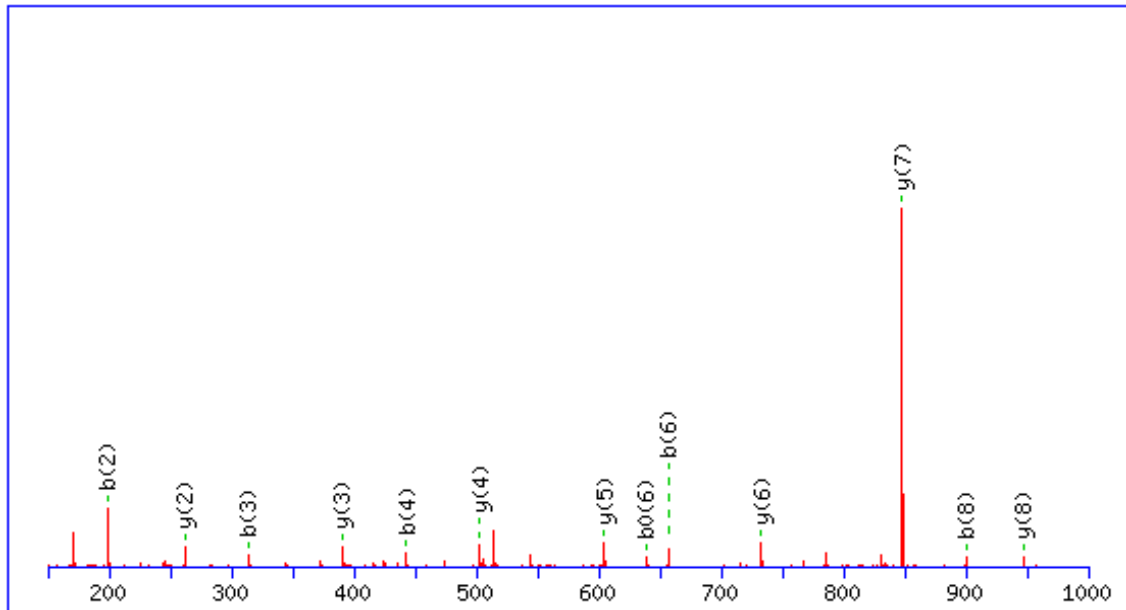
Monoisotopic mass of neutral peptide Mr(calc): 1045.529144

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N3 : Deamidated (NQ)

Ions Score: 65 **Expect:** 0.0029



MS/MS Fragmentation of **LFhSSSALnITELYnVAR**

Found in [gi|151944378](#), glycoprotein [Saccharomyces cerevisiae YJM789]

Monoisotopic mass of neutral peptide Mr(calc): 2013.005432

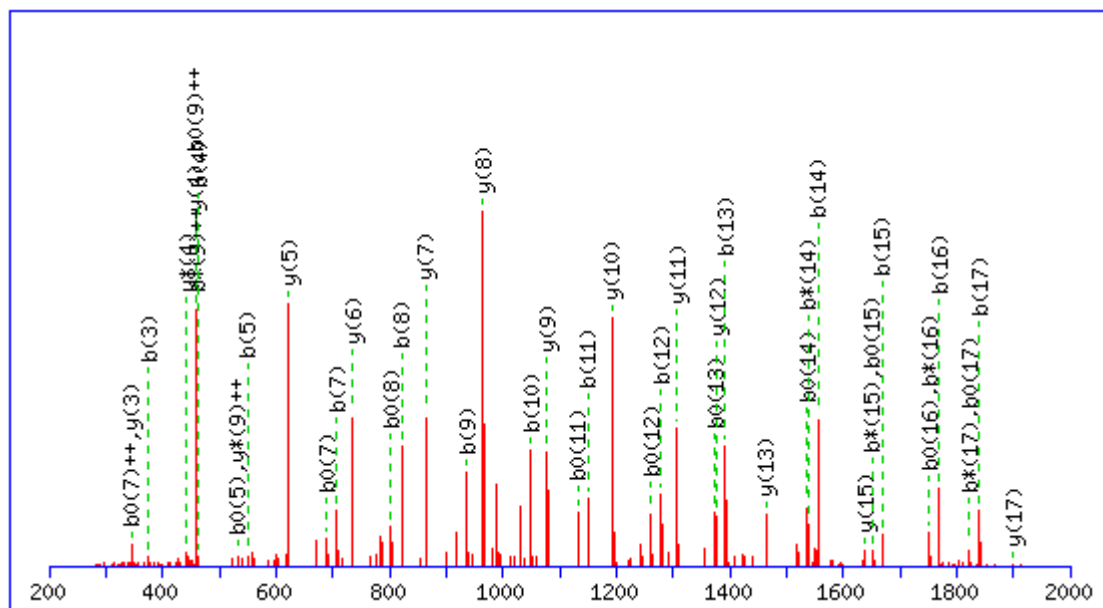
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N3 : Deamidated (NQ)

N9 : Deamidated (NQ)

Ions Score: 113 **Expect:** 7.6e-08



MS/MS Fragmentation of **VQYYKDYCSQLGVSPGDnLTC**

Found in **gi|164699029**, class IV chitinase [*Vitis pseudoreticulata*]

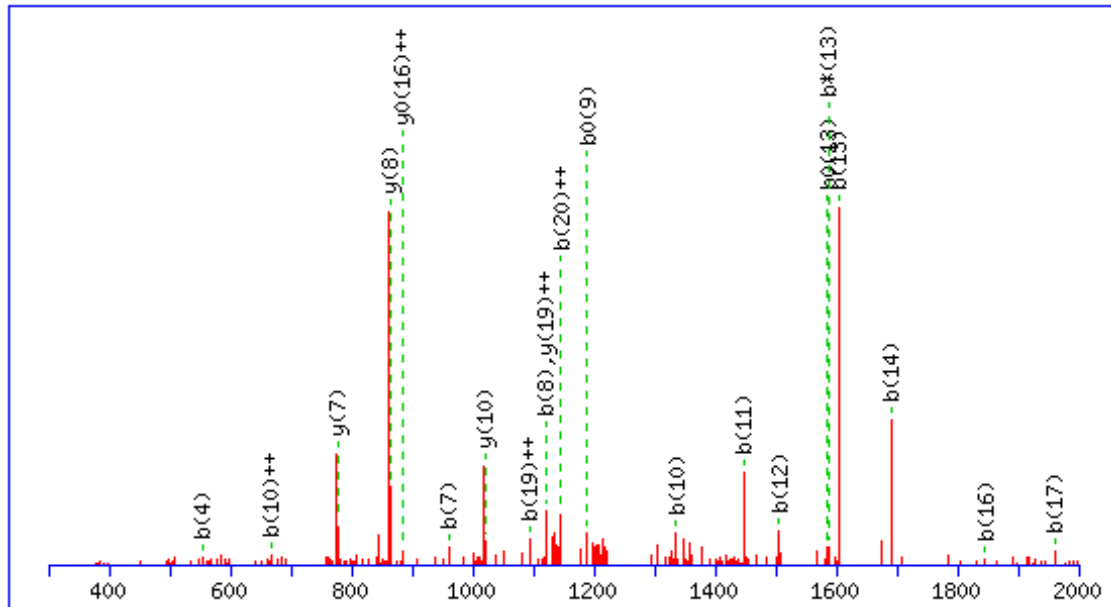
Monoisotopic mass of neutral peptide Mr(calc): 2467.067184

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N18 : Deamidated (NQ)

Ions Score: 56 **Expect:** 0.058



MS/MS Fragmentation of **FDSSSnFSCNALK**

Found in **gi|207347722**, YBR078Wp-like protein [*Saccharomyces cerevisiae* AWRI1631]

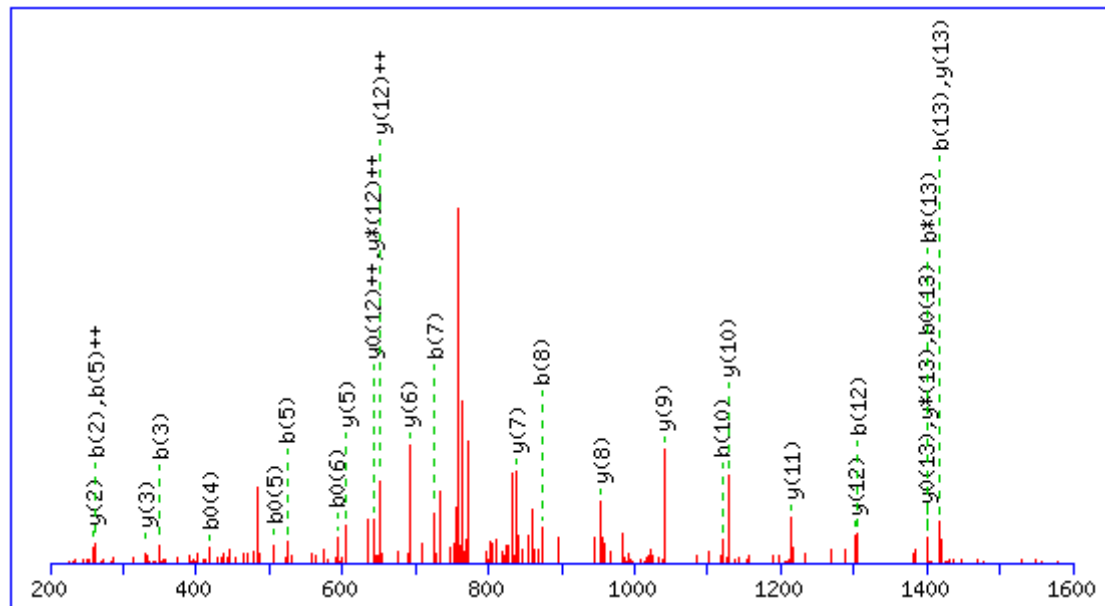
Monoisotopic mass of neutral peptide Mr(calc): 1563.651123

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N7 : Deamidated (NQ)

Ions Score: 81 **Expect:** 7.3e-05



MS/MS Fragmentation of **VYAI nTTLDHSECMK**

Found in **gi|3730**, glycolipid-anchored surface protein [Saccharomyces cerevisiae]

Monoisotopic mass of neutral peptide Mr(calc): 1797.791321

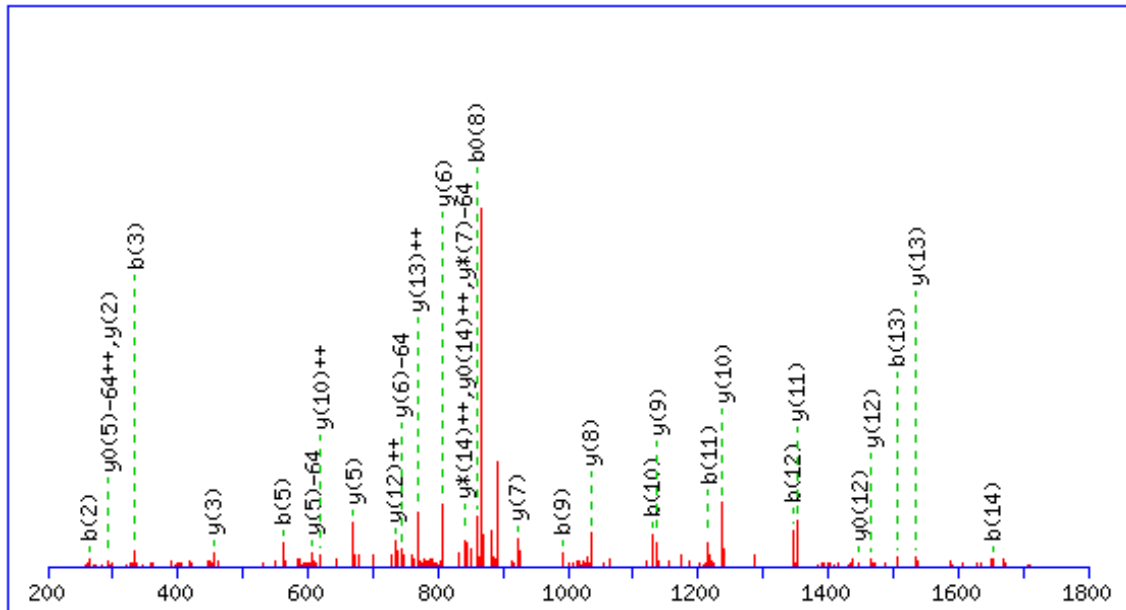
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N5 : Deamidated (NQ)

M14 : Oxidation (M), with neutral losses 0.000000(shown in table), 63.998285

Ions Score: 65 **Expect:** 0.0043



MS/MS Fragmentation of **FFYSNnGSQFYIR**

Found in **gi|3730**, glycolipid-anchored surface protein [*Saccharomyces cerevisiae*]

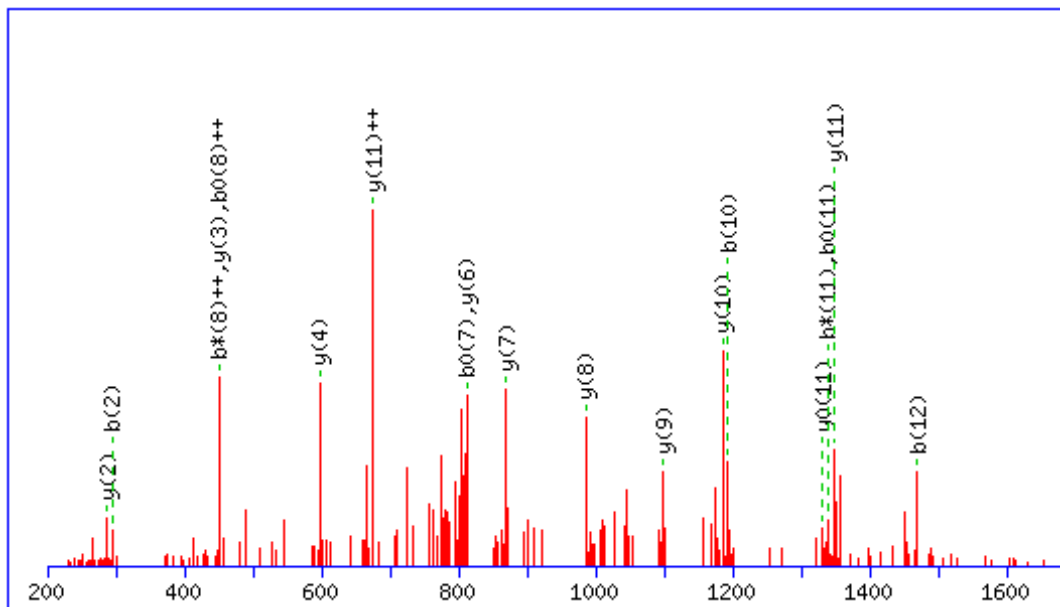
Monoisotopic mass of neutral peptide Mr(calc): 1642.741592

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

Ions Score: 72 **Expect:** 0.0008



MS/MS Fragmentation of **GVAYQADTAnETSGSTVNDPLANYESCSR**

Found in **gi|3730**, glycolipid-anchored surface protein [*Saccharomyces cerevisiae*]

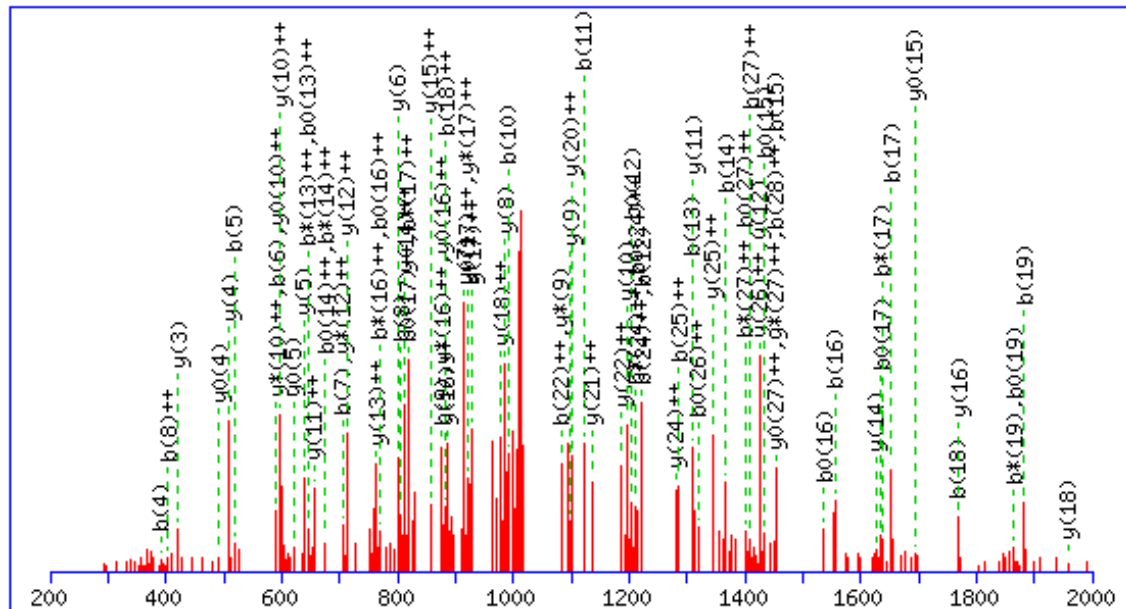
Monoisotopic mass of neutral peptide Mr(calc): 3077.315598

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N10 : Deamidated (NQ)

Ions Score: 94 Expect: 9.9e-06



MS/MS Fragmentation of MNYNVTER

Found in [gi|6323648](#), Phospholipase B (lysophospholipase) involved in phospholipid metabolism; displays transacylase activity in vitro; overproduction confers resistance to lysophosphatidylcholine; Plb2p [Saccharomyces cerevisiae]

Monoisotopic mass of neutral peptide Mr(calc): 1042.438934

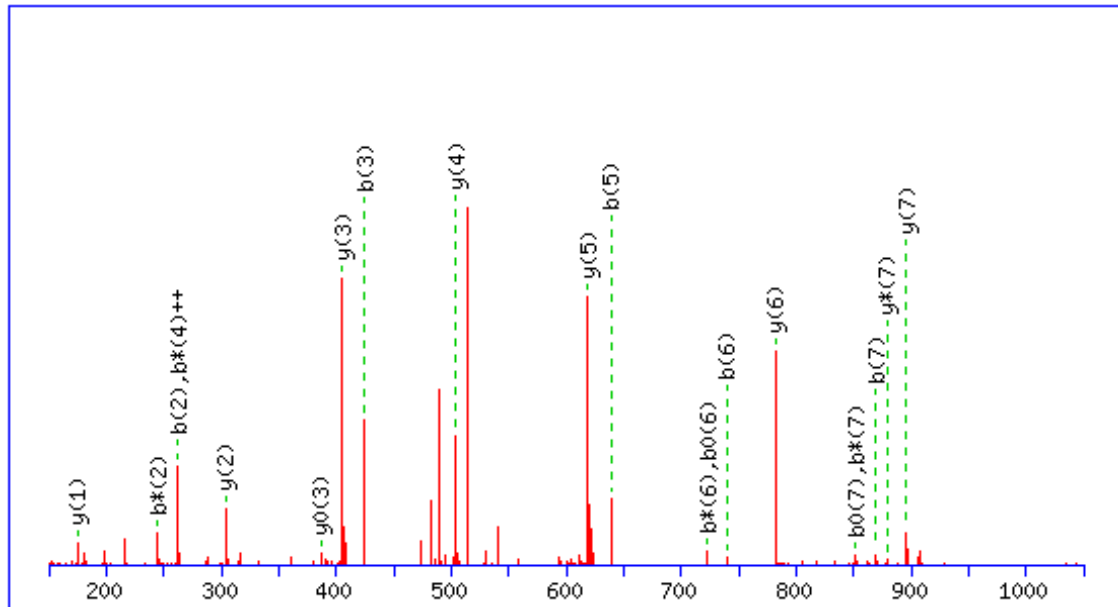
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

M1 : Oxidation (M), with neutral losses 0.000000(shown in table), 63.998285

N4 : Deamidated (NQ)

Ions Score: 50 Expect: 0.055



MS/MS Fragmentation of **HSFNGnQSTLK**

Found in [gi|6323648](#), Phospholipase B (lysophospholipase) involved in phospholipid metabolism; displays transacylase activity in vitro; overproduction confers resistance to lysophosphatidylcholine; Plb2p [*Saccharomyces cerevisiae*]

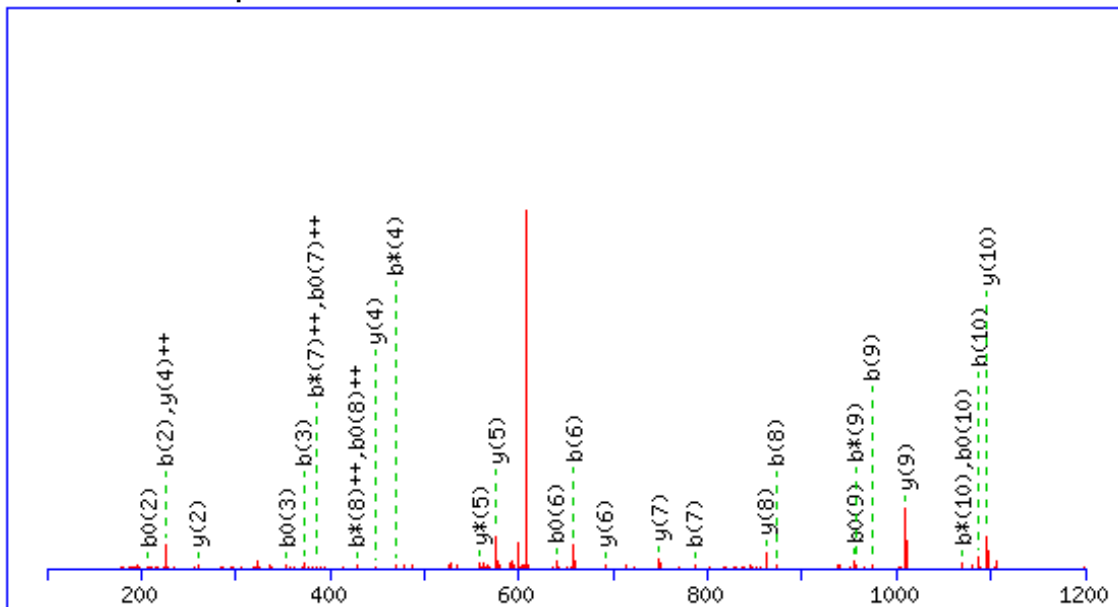
Monoisotopic mass of neutral peptide Mr(calc): 1232.578552

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

Ions Score: 66 **Expect:** 0.002



MS/MS Fragmentation of **YLGtnVTNGKPVNK**

Found in **gi|6323648**, Phospholipase B (lysophospholipase) involved in phospholipid metabolism; displays transacylase activity in vitro; overproduction confers resistance to lysophosphatidylcholine; Plb2p [*Saccharomyces cerevisiae*]

Monoisotopic mass of neutral peptide Mr(calc): 1505.772552

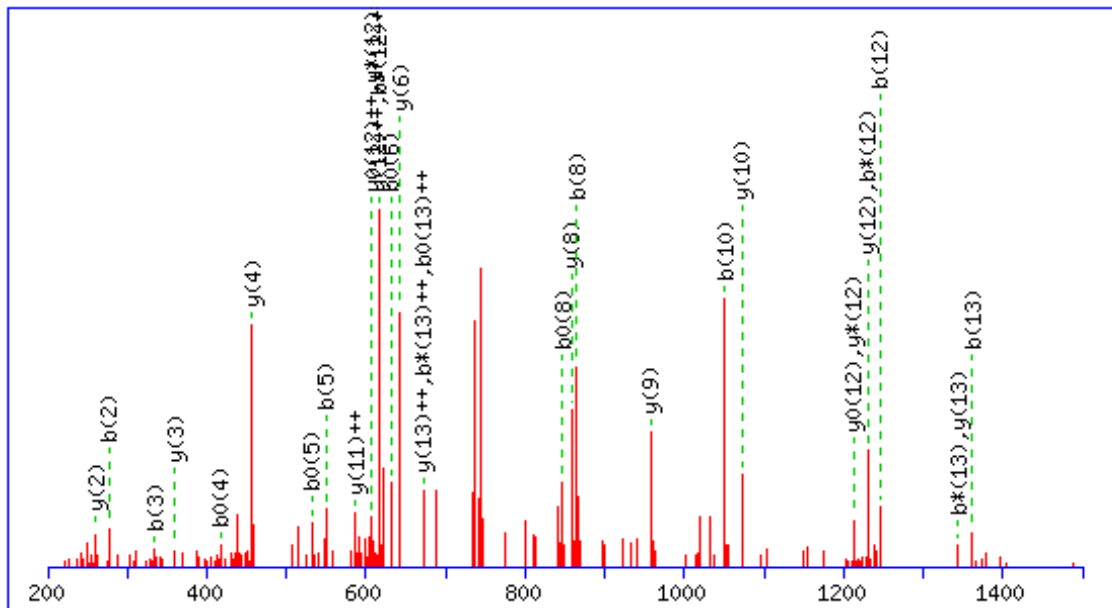
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N5 : Deamidated (NQ)

N8 : Deamidated (NQ)

Ions Score: 72 **Expect:** 0.0008



MS/MS Fragmentation of **QFAFYTSPGFTVnNSR**

Found in **gi|6322290**, GPI-anchored cell wall protein of unknown function; induced in response to cell wall damaging agents and by mutations in genes involved in cell wall biogenesis; sequence similarity to YBR162C/TOS1, a covalently bound cell wall protein; Yjl171cp [*Saccharomyces Cerevisiae*]

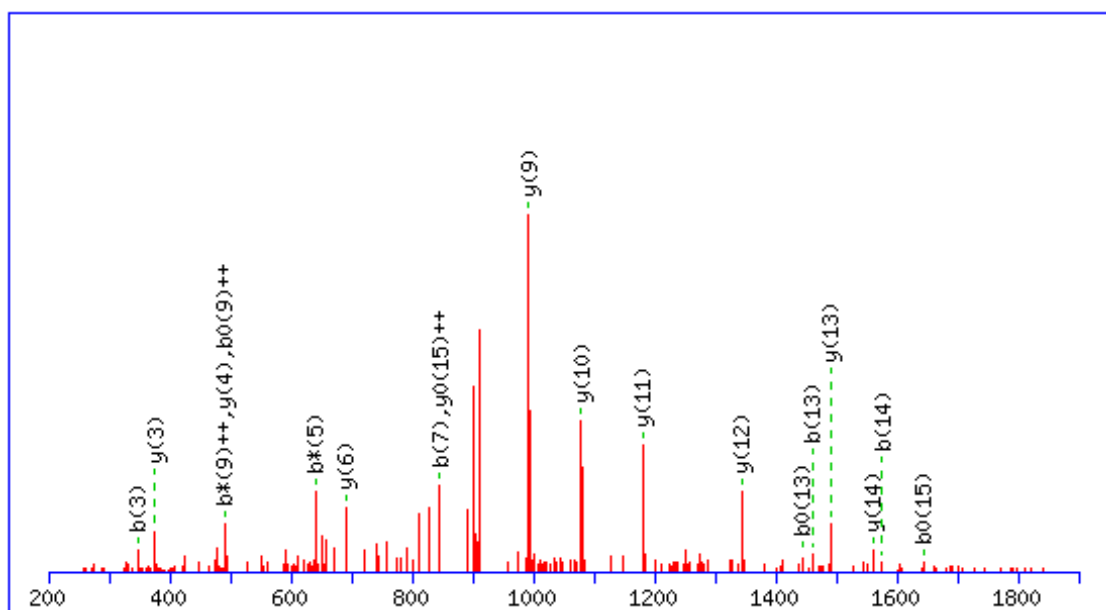
Monoisotopic mass of neutral peptide Mr(calc): 1835.847870

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N13 : Deamidated (NQ)

Ions Score: 79 **Expect:** 0.0002



MS/MS Fragmentation of **LAVnVSAAR**

Found in **gi|225445553**, PREDICTED: hypothetical protein [Vitis vinifera]

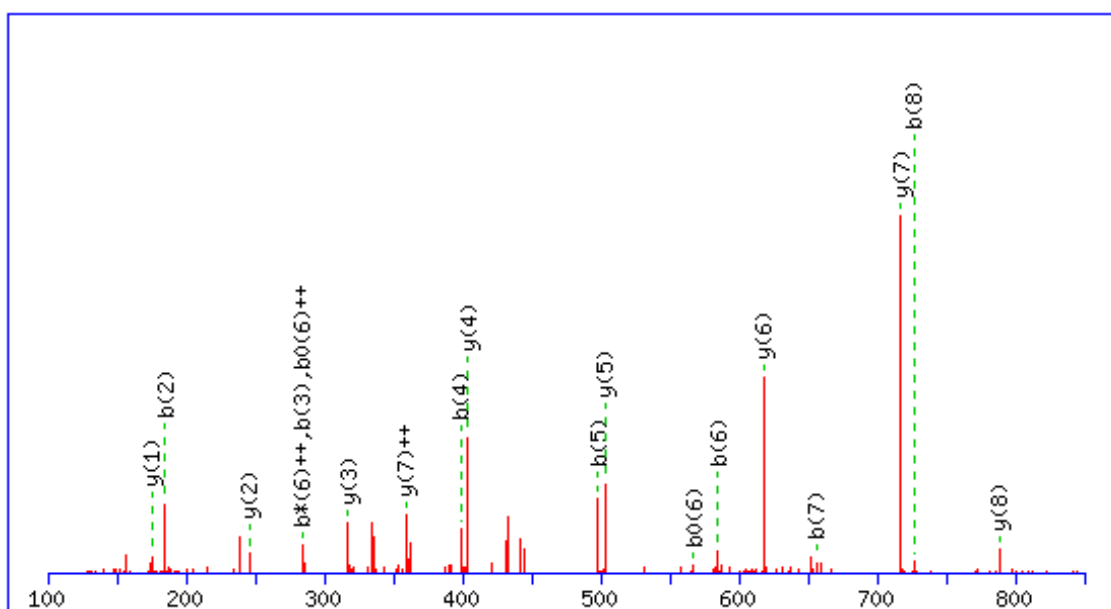
Monoisotopic mass of neutral peptide Mr(calc): 900.502853

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N4 : Deamidated (NQ)

Ions Score: 66 **Expect:** 0.0027



MS/MS Fragmentation of **FnFTTGAHDVAEVTK**

Found in **gi|225445553**, PREDICTED: hypothetical protein [Vitis vinifera]

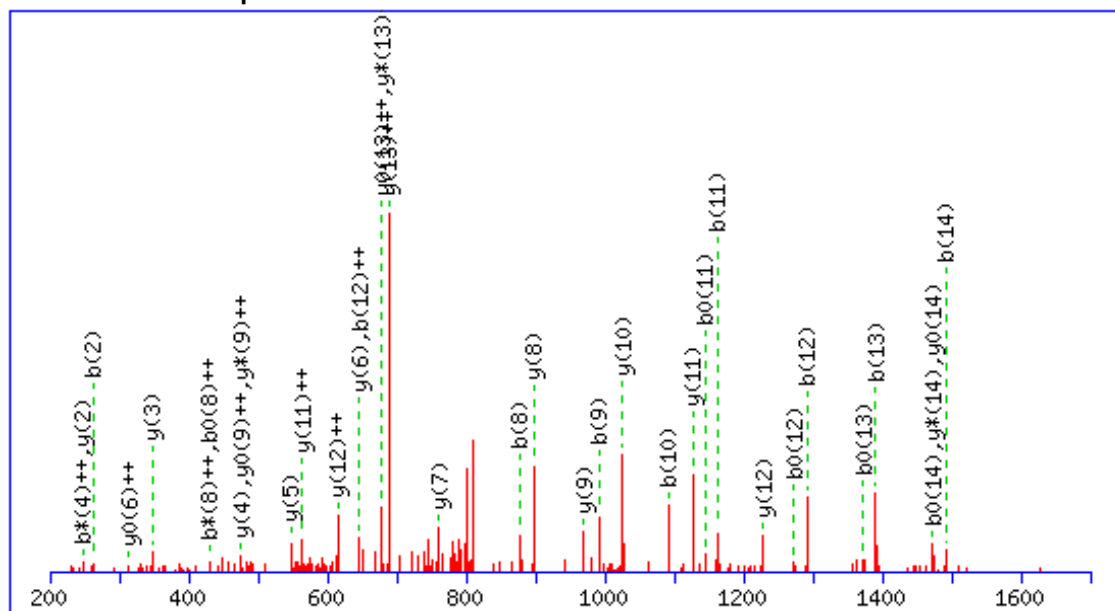
Monoisotopic mass of neutral peptide Mr(calc): 1636.773315

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N2 : Deamidated (NQ)

Ions Score: 96 **Expect:** 4.3e-06



MS/MS Fragmentation of **AAFTACnGTNPISHETEGPADIDLDTAGEHYFICTVGSHCSLGQK**

Found in **gi|225445553**, PREDICTED: hypothetical protein [Vitis vinifera]

Monoisotopic mass of neutral peptide Mr(calc): 4851.101089

Fixed modifications: Carbamidomethyl (C)

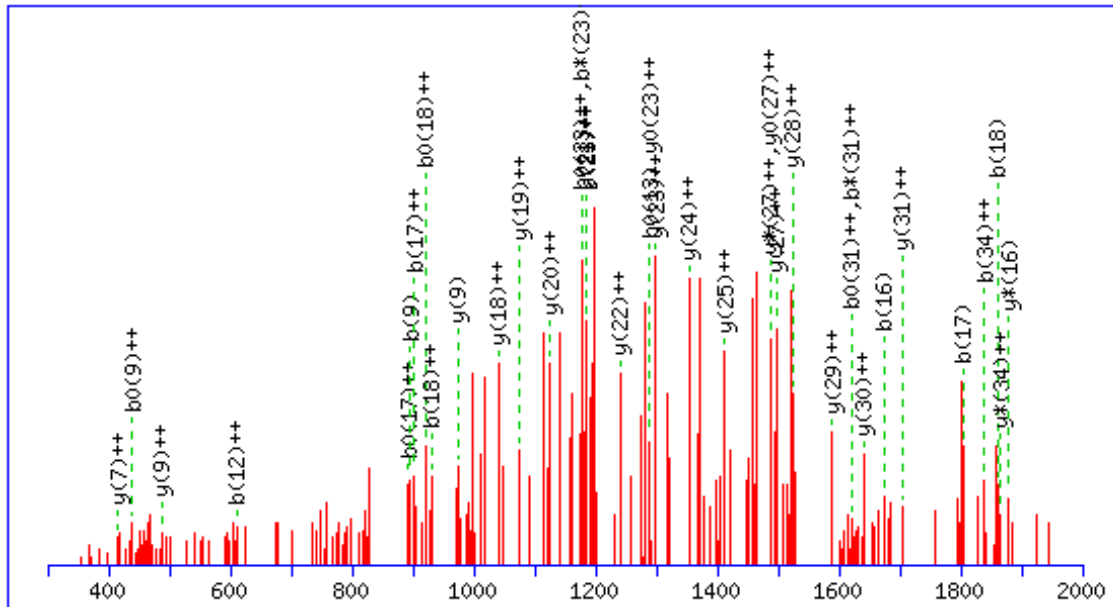
Variable modifications:

N7 : Deamidated (NQ)

N10 : Deamidated (NQ)

Q44 : Deamidated (NQ)

Ions Score: 32 **Expect:** 19



MS/MS Fragmentation of **FYDEdGCQYnGSFDGMK**

Found in [gi|225454402](#), PREDICTED: hypothetical protein [Vitis vinifera]

Monoisotopic mass of neutral peptide Mr(calc): 2048.740433

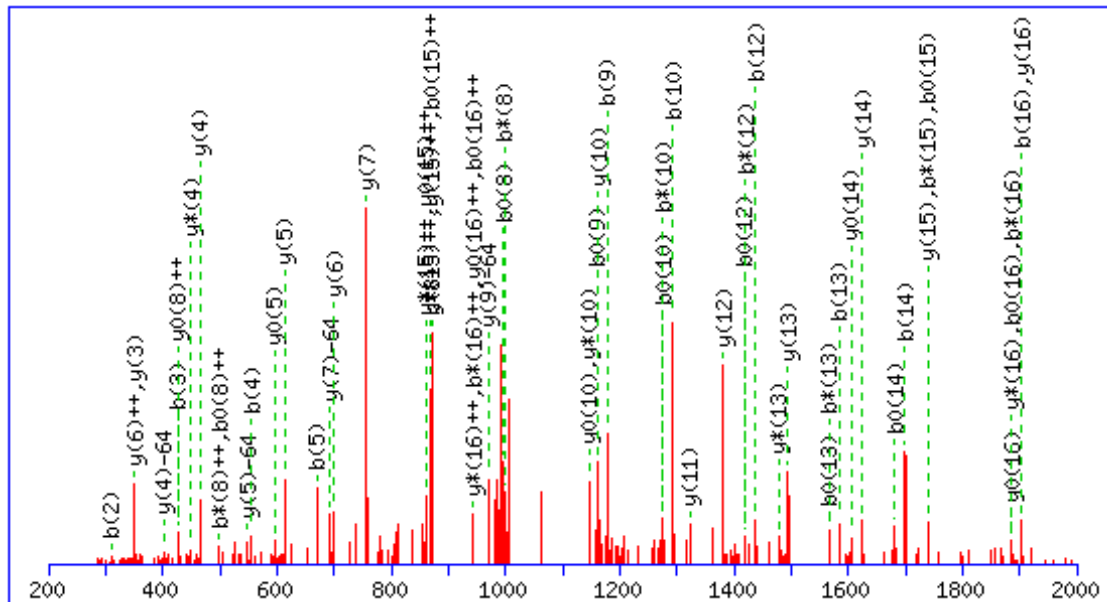
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N10 : Deamidated (NQ)

M16 : Oxidation (M), with neutral losses 0.000000(shown in table), 63.998285

Ions Score: 95 **Expect:** 1.5e-06



MS/MS Fragmentation of **NTNLMLGnVTMQK**

Found in [gi|225459538](#), PREDICTED: hypothetical protein [Vitis vinifera]

Monoisotopic mass of neutral peptide Mr(calc): 1495.701050

Fixed modifications: Carbamidomethyl (C)

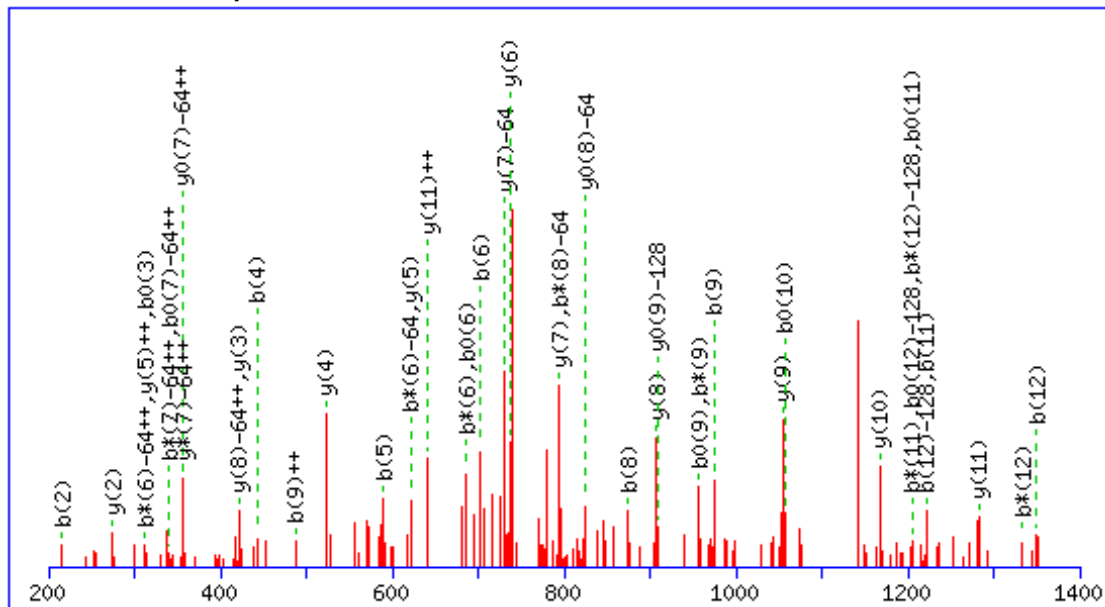
Variable modifications:

M5 : Oxidation (M), with neutral losses 0.000000(shown in table),
63.998285

N8 : Deamidated (NQ)

M11 : Oxidation (M), with neutral losses 0.000000(shown in table),
63.998285

Ions Score: 70 Expect: 0.0012

MS/MS Fragmentation of **HSFNGnQSTFK**

Found in [gi|437732](#), lysophospholipase

Monoisotopic mass of neutral peptide Mr(calc): 1267.546921

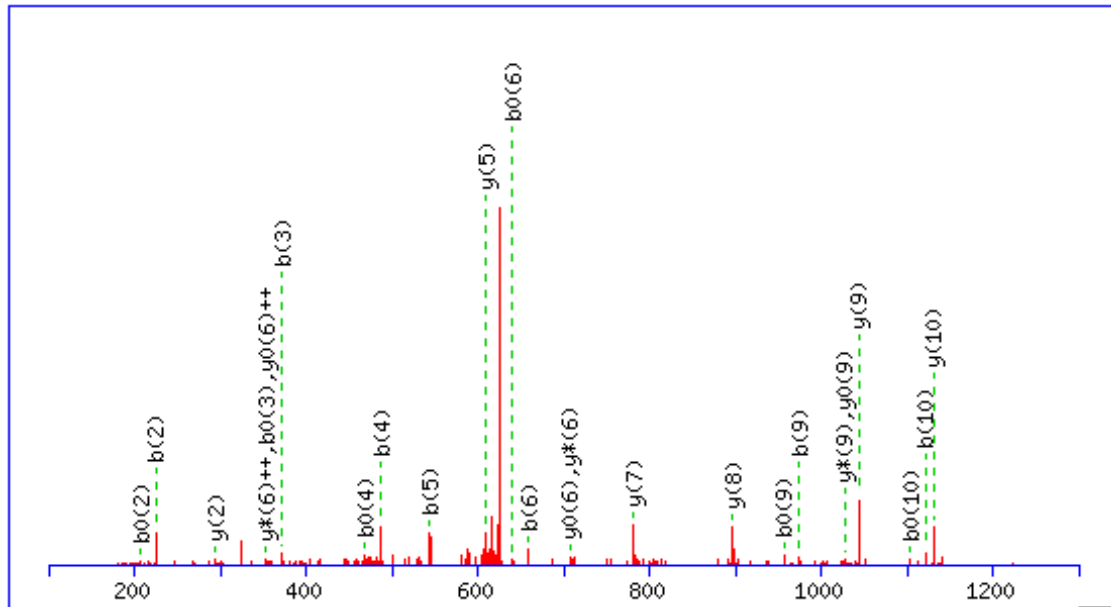
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N4 : Deamidated (NQ)

N6 : Deamidated (NQ)

Ions Score: 35 Expect: 2.4



MS/MS Fragmentation of IISFnLSDAETGK

Found in [gi|82795241](#), Vacuolar aminopeptidase Y, processed to mature form by Prb1p; Ape3p [Saccharomyces cerevisiae]

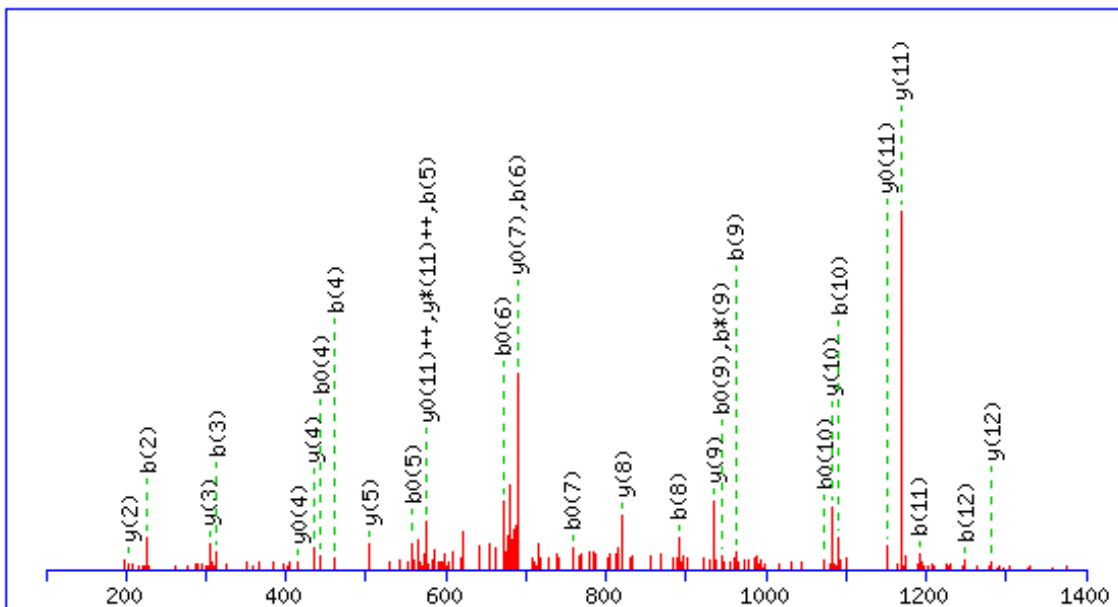
Monoisotopic mass of neutral peptide Mr(calc): 1394.692902

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N5 : Deamidated (NQ)

Ions Score: 71 **Expect:** 0.0013



MS/MS Fragmentation of **LANYSTPDYGHPT**R

Found in **gi|82795241**, Vacuolar aminopeptidase Y, processed to mature form by Prb1p; Ape3p
[Saccharomyces cerevisiae]

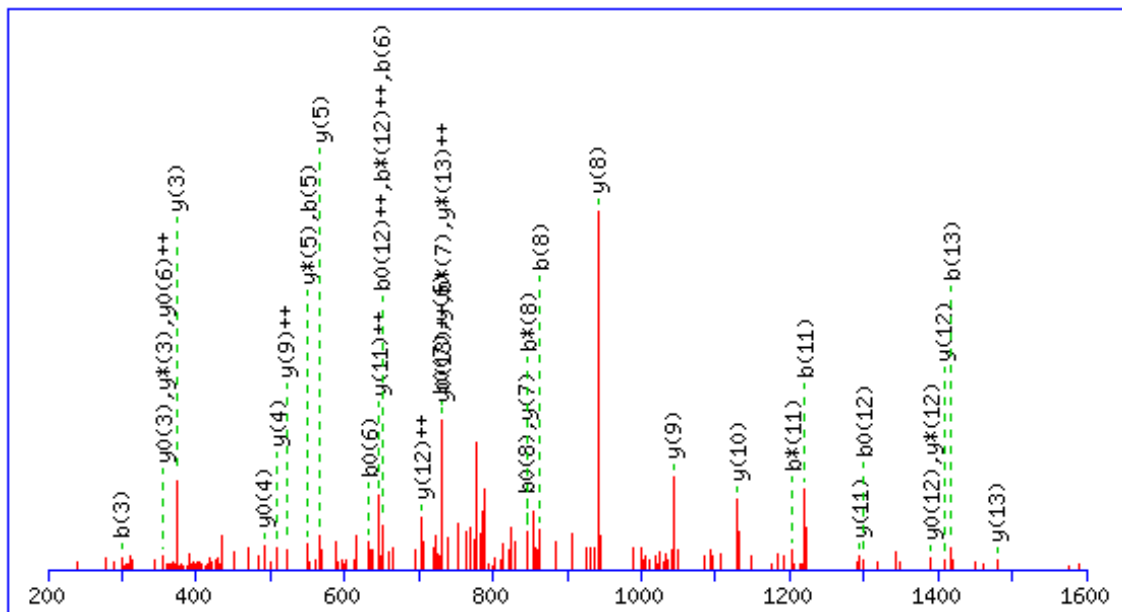
Monoisotopic mass of neutral peptide Mr(calc): 1591.726669

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N3 : Deamidated (NQ)

Ions Score: 61 **Expect:** 0.01



MS/MS Fragmentation of **LSnYTR**

Found in **gi|758281**, constitutive acid phosphatase (PH03) [Saccharomyces cerevisiae]

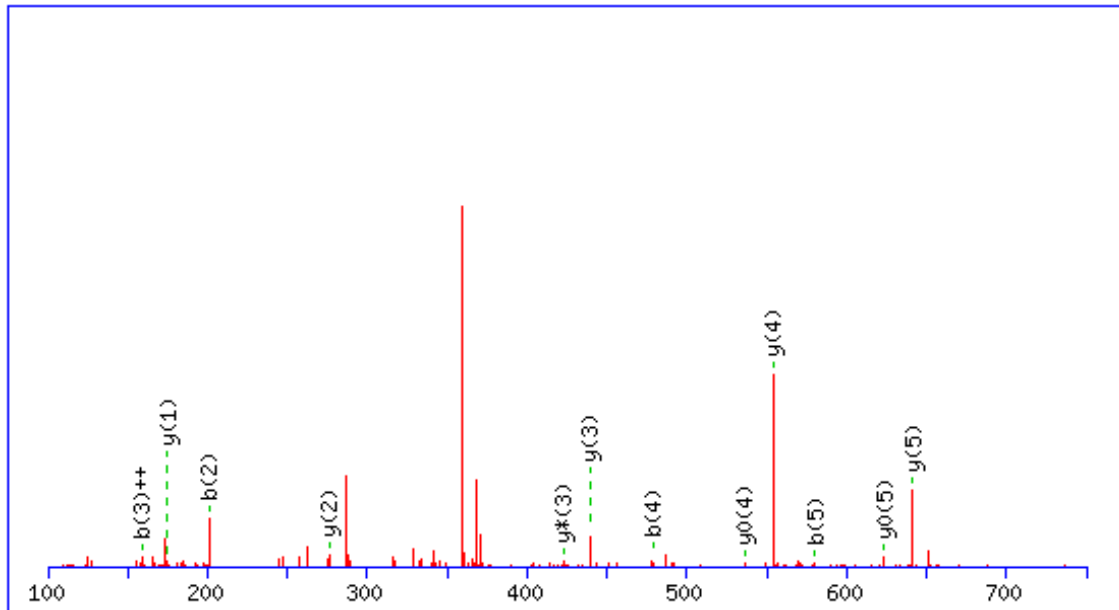
Monoisotopic mass of neutral peptide Mr(calc): 753.365692

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N3 : Deamidated (NQ)

Ions Score: 26 **Expect:** 14



MS/MS Fragmentation of **FQCSnDTYVR**

Found in **gi|758281**, constitutive acid phosphatase (PH03) [*Saccharomyces cerevisiae*]

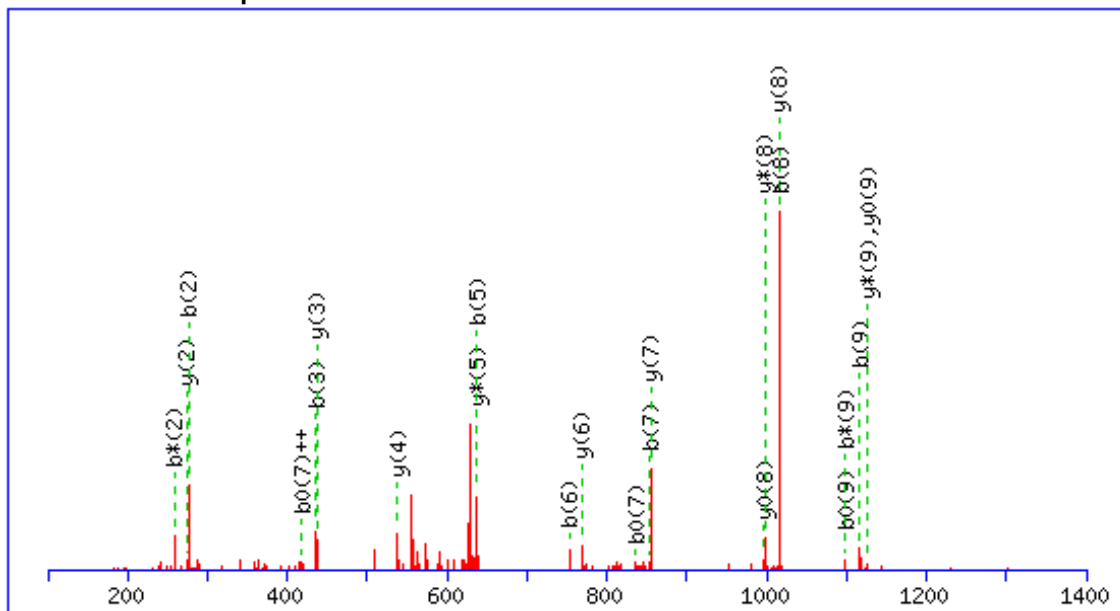
Monoisotopic mass of neutral peptide Mr(calc): 1289.534653

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N5 : Deamidated (NQ)

Ions Score: 40 **Expect:** 0.55



MS/MS Fragmentation of **GNYYVnSTFGTPGQR**

Found in **gi|6320556**, Putative GPI-anchored aspartic protease, located in the cytoplasm and endoplasmic reticulum; Yps7p [*Saccharomyces cerevisiae*]

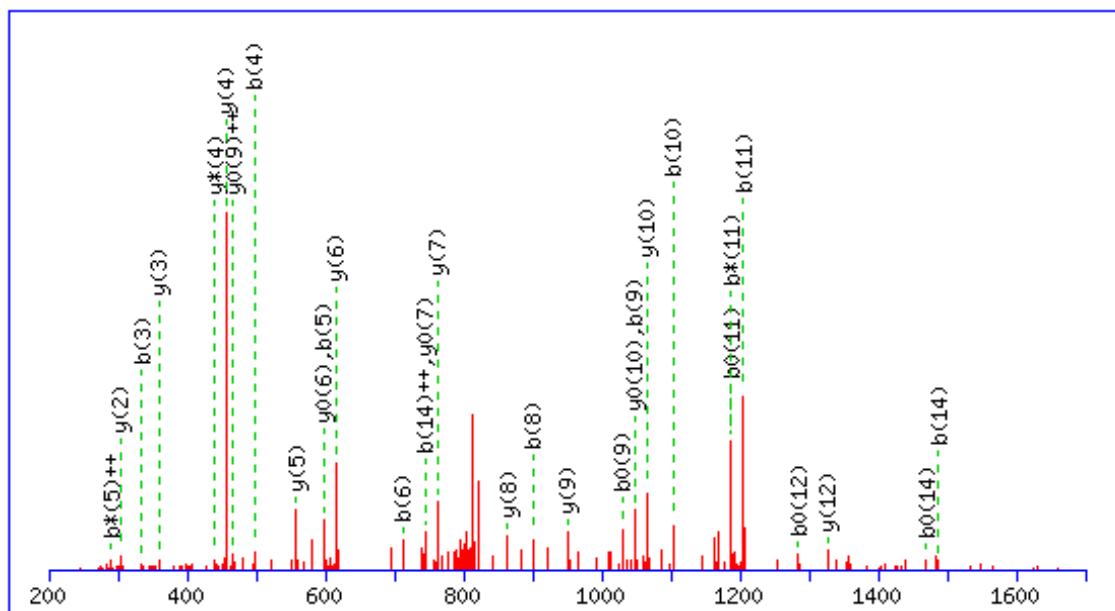
Monoisotopic mass of neutral peptide Mr(calc): 1660.748154

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

Ions Score: 75 Expect: 0.00047



MS/MS Fragmentation of **VATDnTTIVEMDPGSFR**

Found in **gi|225447326**, PREDICTED: hypothetical protein [Vitis vinifera]

Monoisotopic mass of neutral peptide Mr(calc): 1868.846222

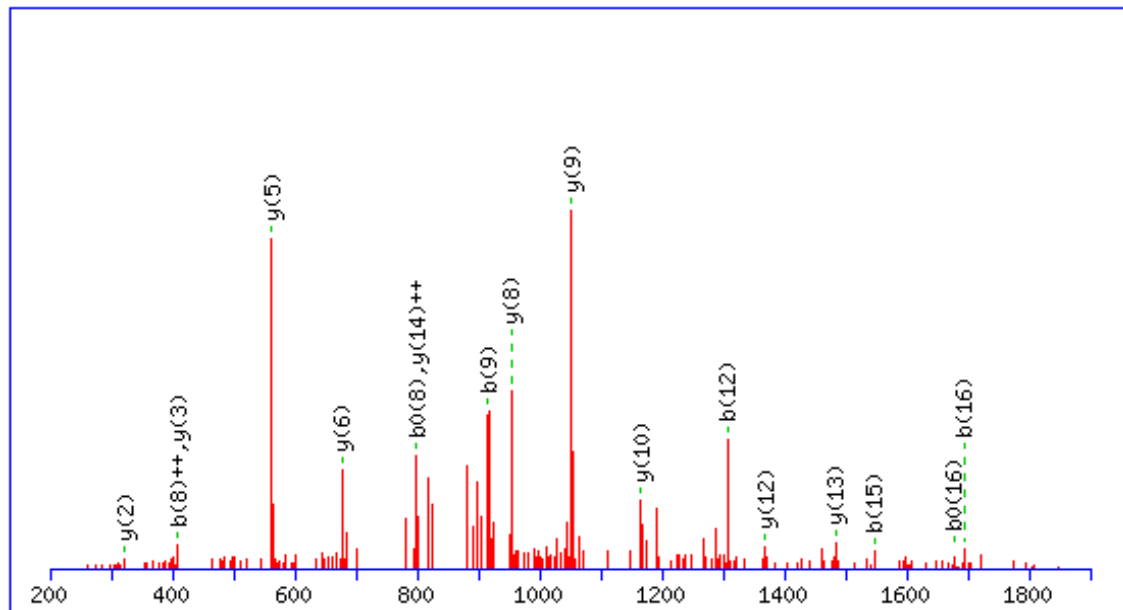
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N5 : Deamidated (NQ)

M11 : Oxidation (M), with neutral losses 0.000000(shown in table), 63.998285

Ions Score: 71 Expect: 0.0015



MS/MS Fragmentation of **VVFDDEDKEYIIIGTALnMTFLK**

Found in **gi|2499716**, RecName: Full=Exopolygalacturonase; Short=ExoPG; AltName: Full=Galacturan 1,4-alpha-galacturonidase; AltName: Full=Poly(1,4-alpha-D-galacturonide)galacturonohydrolase; Flags: Precursor

Monoisotopic mass of neutral peptide Mr(calc): 2462.229065

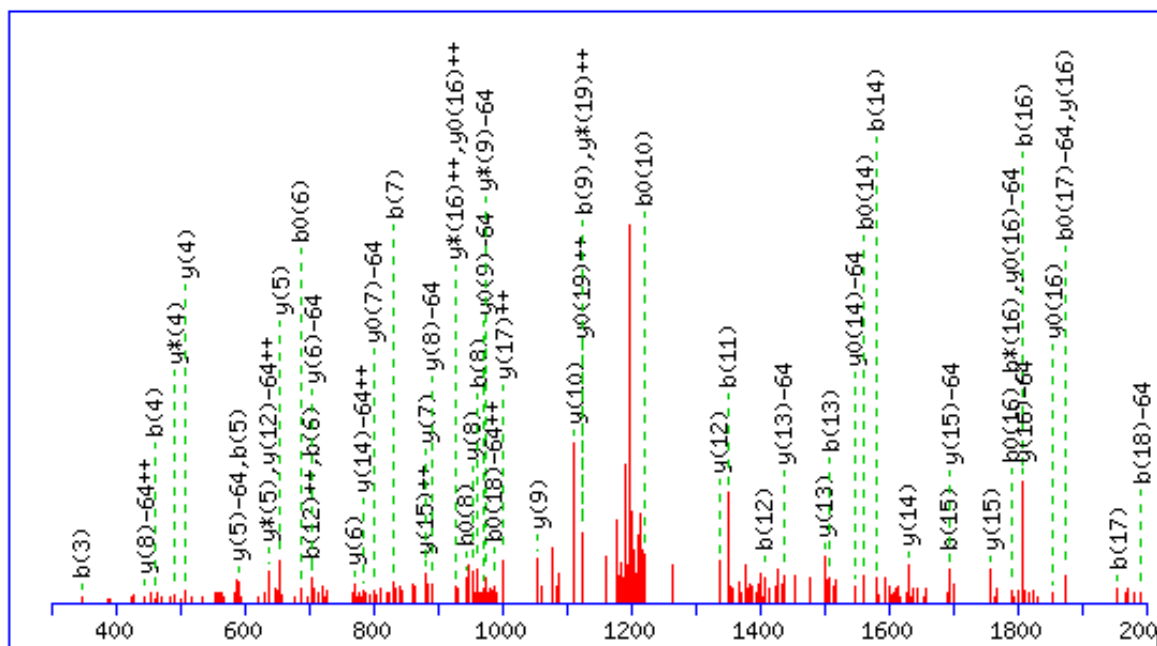
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N16 : Deamidated (NQ)

M17 : Oxidation (M), with neutral losses 63.998285(shown in table), 0.000000

Ions Score: 52 **Expect:** 0.13



MS/MS Fragmentation of **YSPQYYHFVAnSSNVLFDGIDISGYSK**

Found in **gi|2499716**, RecName: Full=Exopolygalacturonase; Short=ExoPG; AltName: Full=Galacturan 1,4-alpha-galacturonidase; AltName: Full=Poly(1,4-alpha-D-galacturonide)galacturonohydrolase; Flags: Precursor

Monoisotopic mass of neutral peptide Mr(calc): 3072.402878

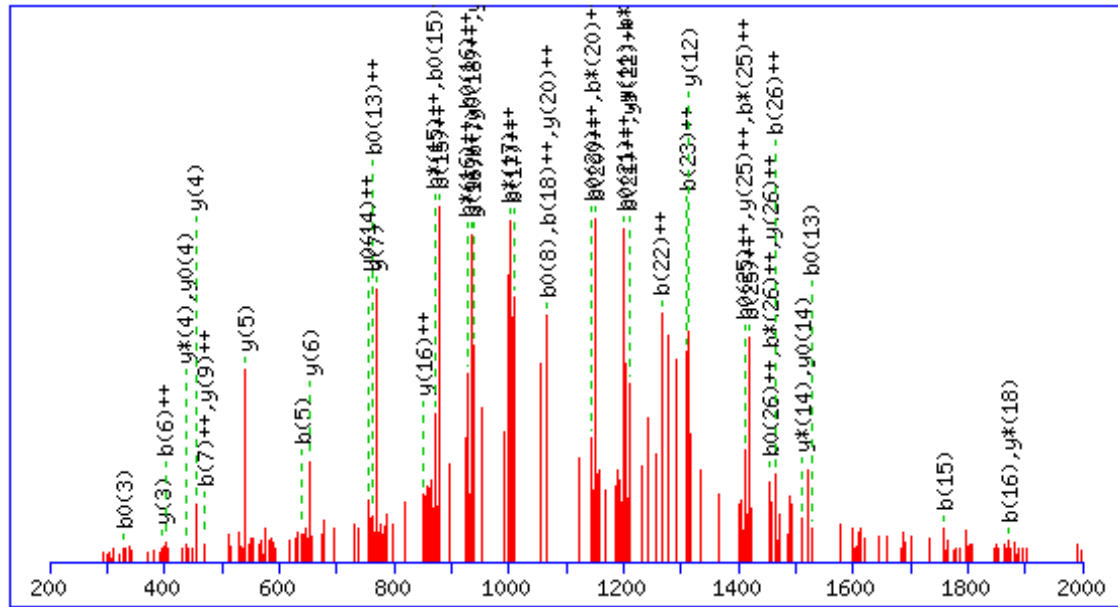
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N11 : Deamidated (NQ)

N14 : Deamidated (NQ)

Ions Score: 34 Expect: 11



MS/MS Fragmentation of **YSnESFFSQVSESPR**

Found in **gi|157357223**, unnamed protein product [Vitis vinifera]

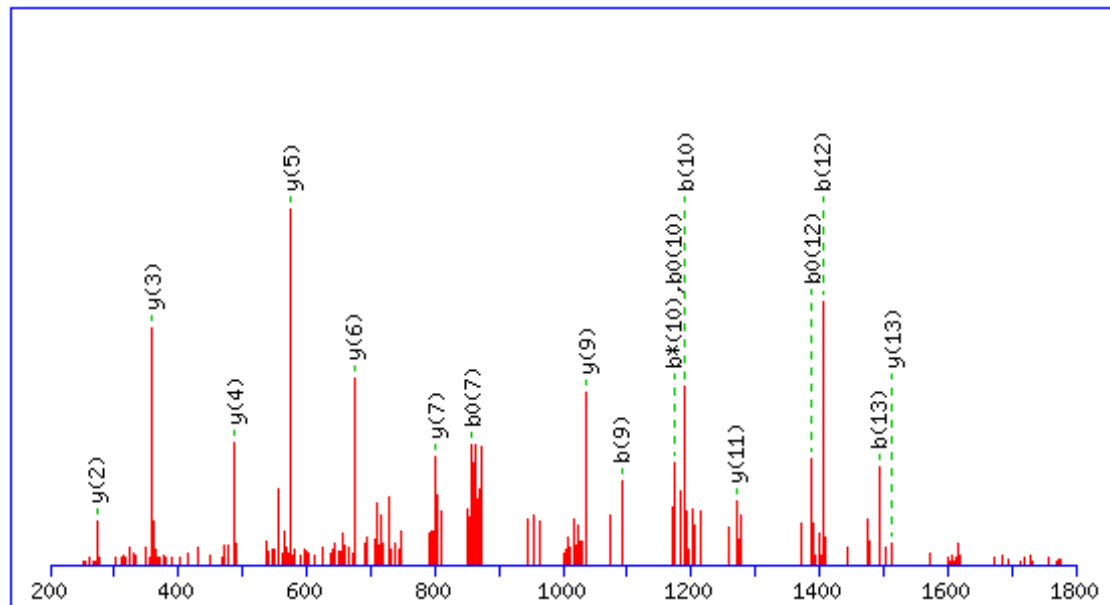
Monoisotopic mass of neutral peptide Mr(calc): 1763.763840

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N3 : Deamidated (NQ)

Ions Score: 64 **Expect:** 0.0054



MS/MS Fragmentation of **LTNSENSVlnDTAPANK**

Found in **gi|147784619**, hypothetical protein [Vitis vinifera]

Monoisotopic mass of neutral peptide Mr(calc): 1788.837708

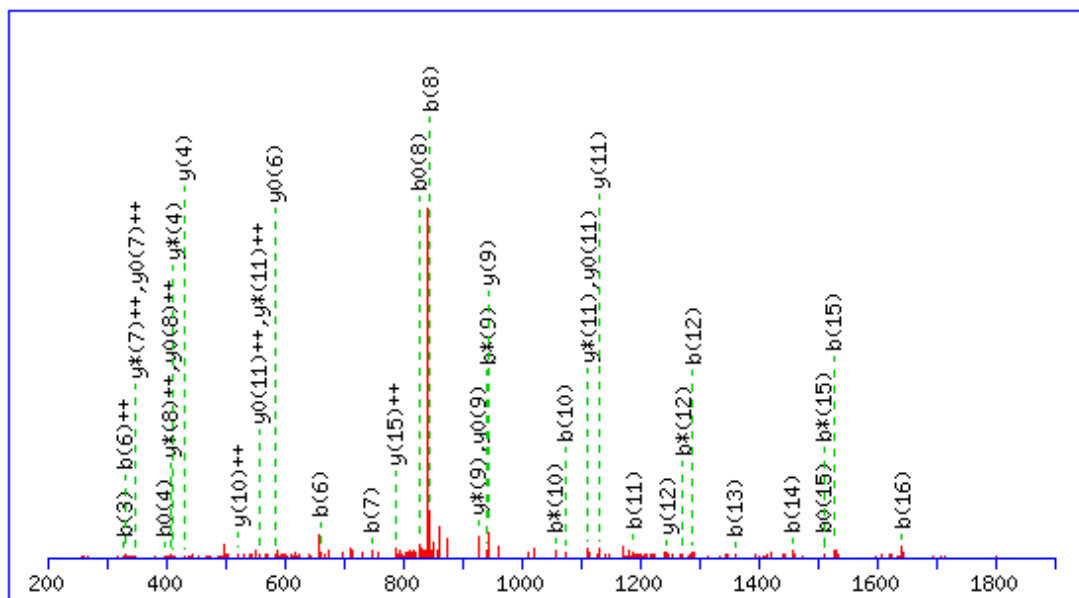
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

N16 : Deamidated (NQ)

Ions Score: 31 Expect: 13



Annotated spectra Titanium dioxide:

MS/MS Fragmentation of **TDEYCCNSGScnATTYSEFFK**

Found in **gi|225426795**, PREDICTED: hypothetical protein [Vitis vinifera]

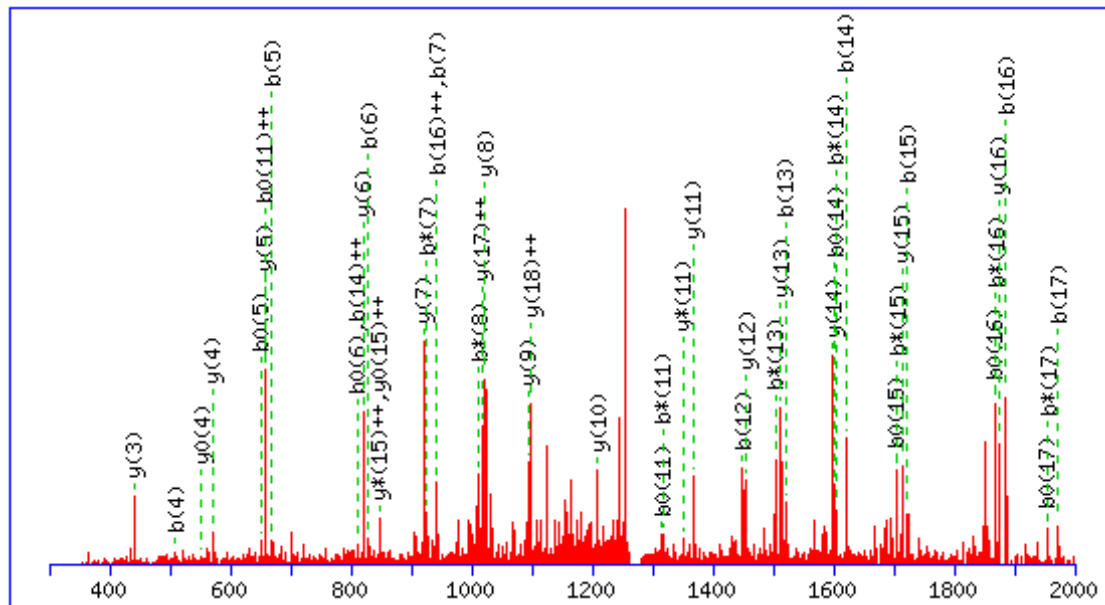
Monoisotopic mass of neutral peptide Mr(calc): 2540.940659

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N12 : Deamidated (NQ)

Ions Score: 74 **Expect:** 0.00031



MS/MS Fragmentation of **AMLGGAGMIAAMDnRT**

Found in **gi|6323648**, Phospholipase B (lysophospholipase) involved in phospholipid metabolism; displays transacylase activity in vitro; overproduction confers resistance to lysophosphatidylcholine; Plb2p [Saccharomyces cerevisiae]

Monoisotopic mass of neutral peptide Mr(calc): 1494.662872

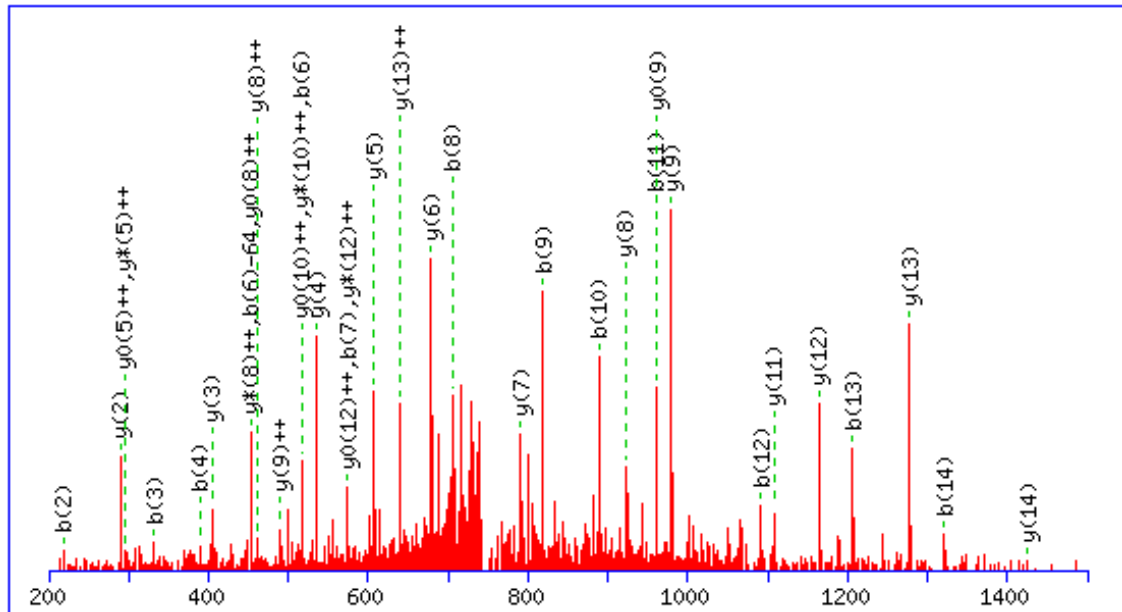
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

M2 : Oxidation (M), with neutral losses 0.000000(shown in table), 63.998285

N14 : Deamidated (NQ)

Ions Score: 91 **Expect:** 8.2e-06



MS/MS Fragmentation of **SIVNPGGSnLTYTIER**

Found in **gi|6323648**, Phospholipase B (lysophospholipase) involved in phospholipid metabolism; displays transacylase activity in vitro; overproduction confers resistance to lysophosphatidylcholine; Plb2p [*Saccharomyces cerevisiae*]

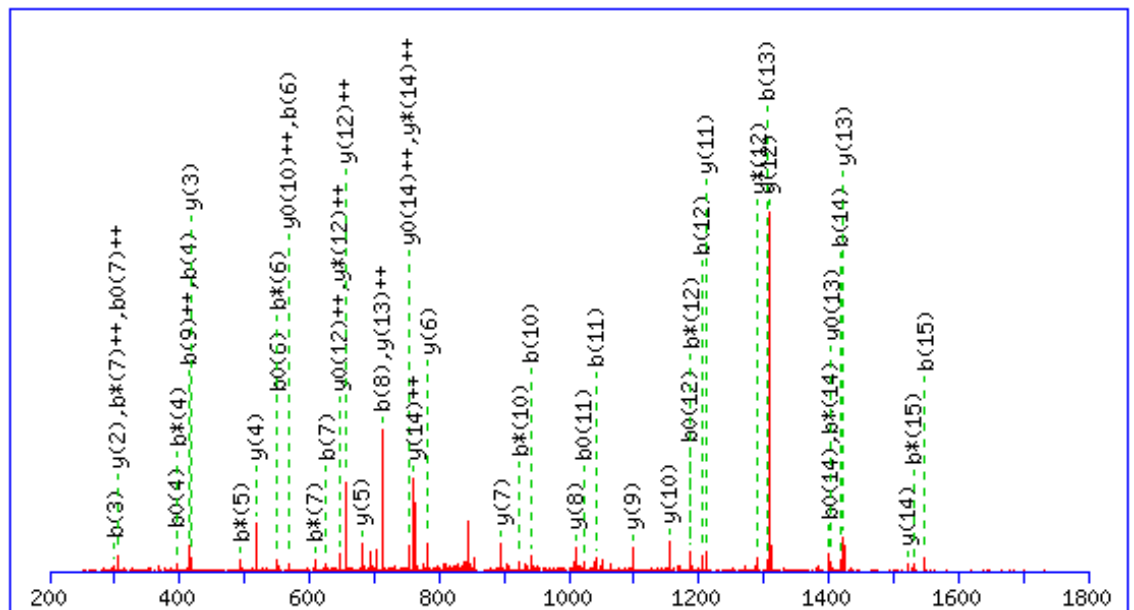
Monoisotopic mass of neutral peptide Mr(calc): 1720.863159

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N9 : Deamidated (NQ)

Ions Score: 89 **Expect:** 1.3e-05



MS/MS Fragmentation of **LFLFNnATGVnVTASIK**

Found in **gi|1839578**, vacuolar invertase 1, GIN1 [Vitis vinifera=grape berries, Sultana, berries, Peptide, 642 aa]

Monoisotopic mass of neutral peptide Mr(calc): 1809.951248

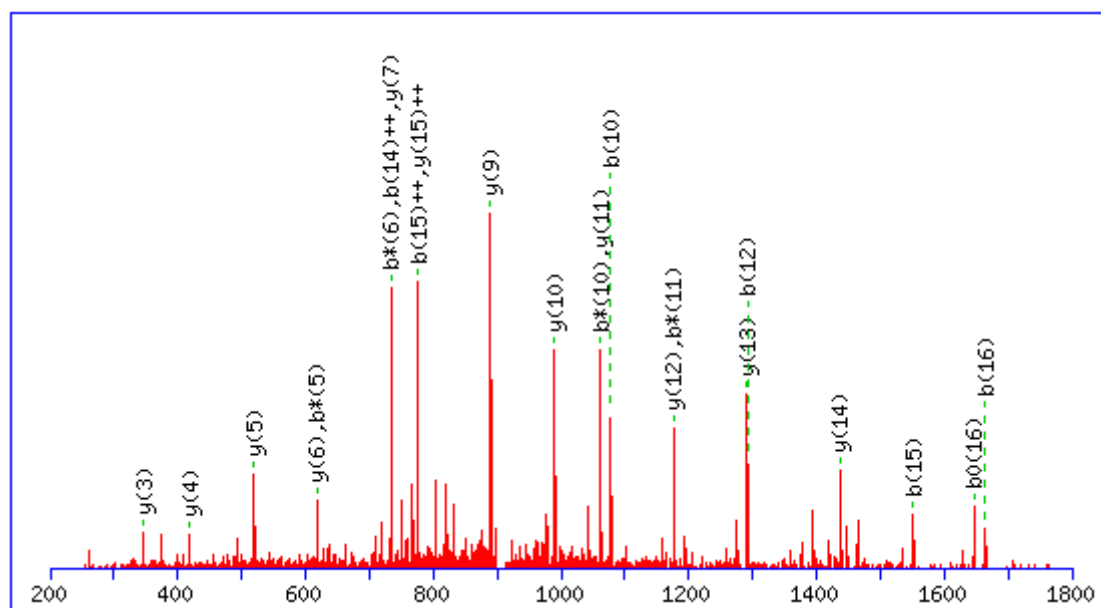
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

N11 : Deamidated (NQ)

Ions Score: 109 **Expect:** 8.7e-08



MS/MS Fragmentation of **TAEFKnLSIPVFFSEYGCNEVTPR**

Found in **gi|3730**, glycolipid-anchored surface protein [*Saccharomyces cerevisiae*]

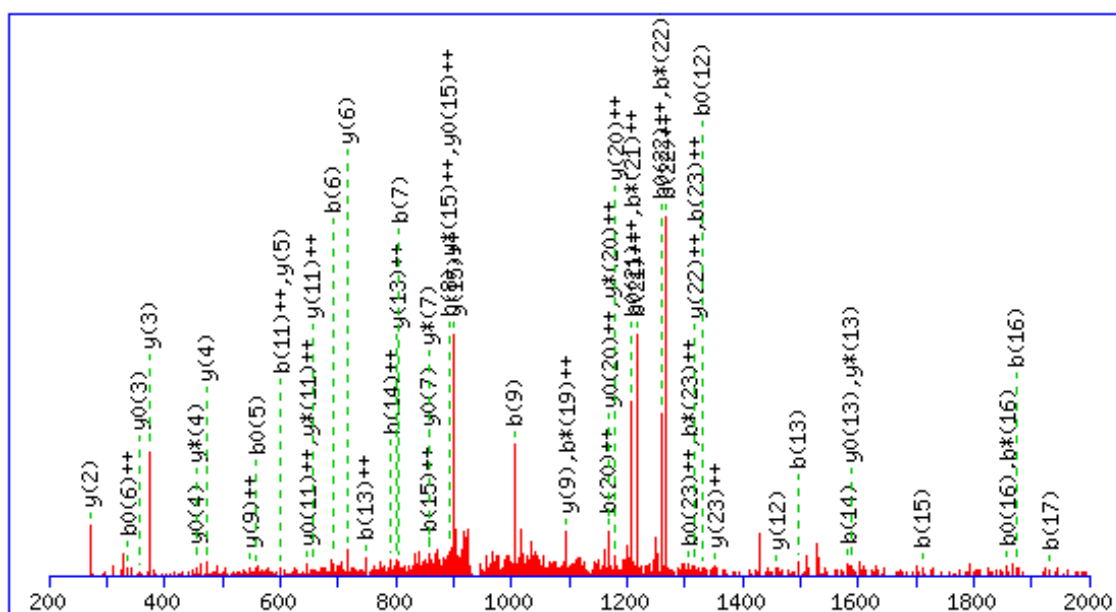
Monoisotopic mass of neutral peptide Mr(calc): 2805.331970

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

Ions Score: 22 **Expect:** 1.3e+02



MS/MS Fragmentation of FFYSNnGSQFYIR

Found in [gi|3730](#), glycolipid-anchored surface protein [Saccharomyces cerevisiae]

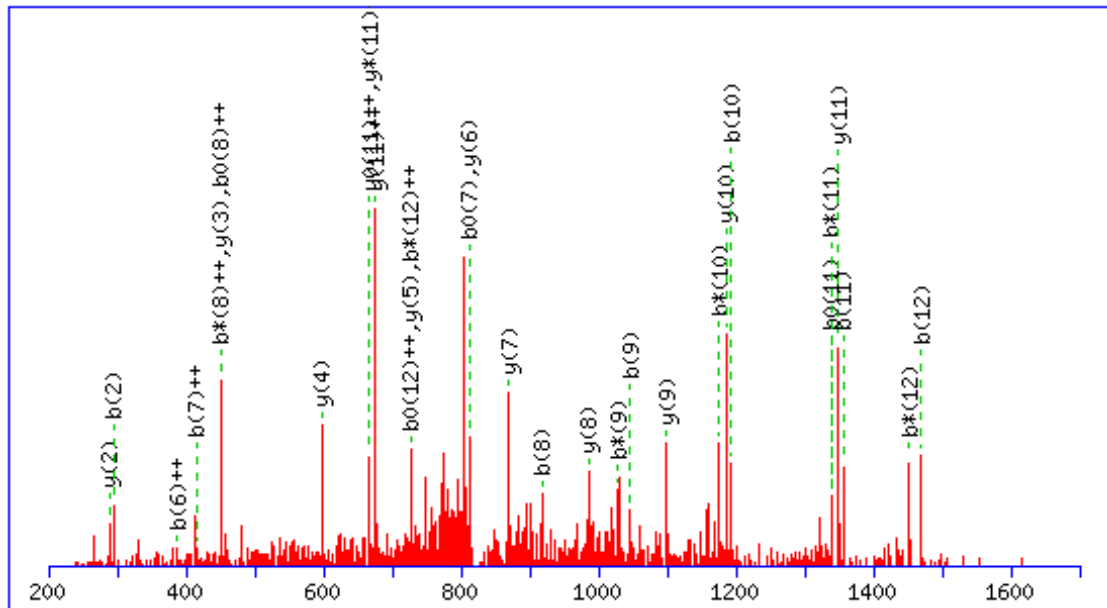
Monoisotopic mass of neutral peptide Mr(calc): 1642.741592

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N6 : Deamidated (NQ)

Ions Score: 66 **Expect:** 0.0028



MS/MS Fragmentation of GDTTTYDRGEFHGVDTPTDKFHnYTLDWAMDK

Found in [gi|6321628](#), Cell wall protein that functions in the transfer of chitin to beta(1-6)glucan, putative chitin transglycosidase; Crh1p [Saccharomyces cerevisiae]

Monoisotopic mass of neutral peptide Mr(calc): 3749.621674

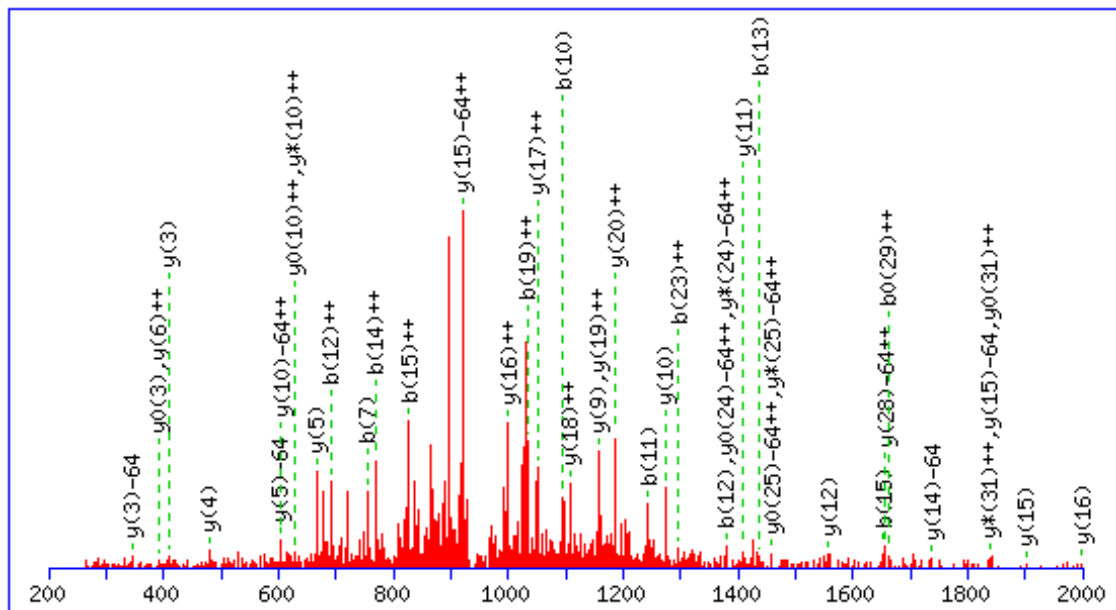
Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N23 : Deamidated (NQ)

M30 : Oxidation (M), with neutral losses 0.000000(shown in table), 63.998285

Ions Score: 26 **Expect:** 70



MS/MS Fragmentation of **VVnETIQDK**

Found in **gi|297485**, GP38 [*Saccharomyces cerevisiae*]

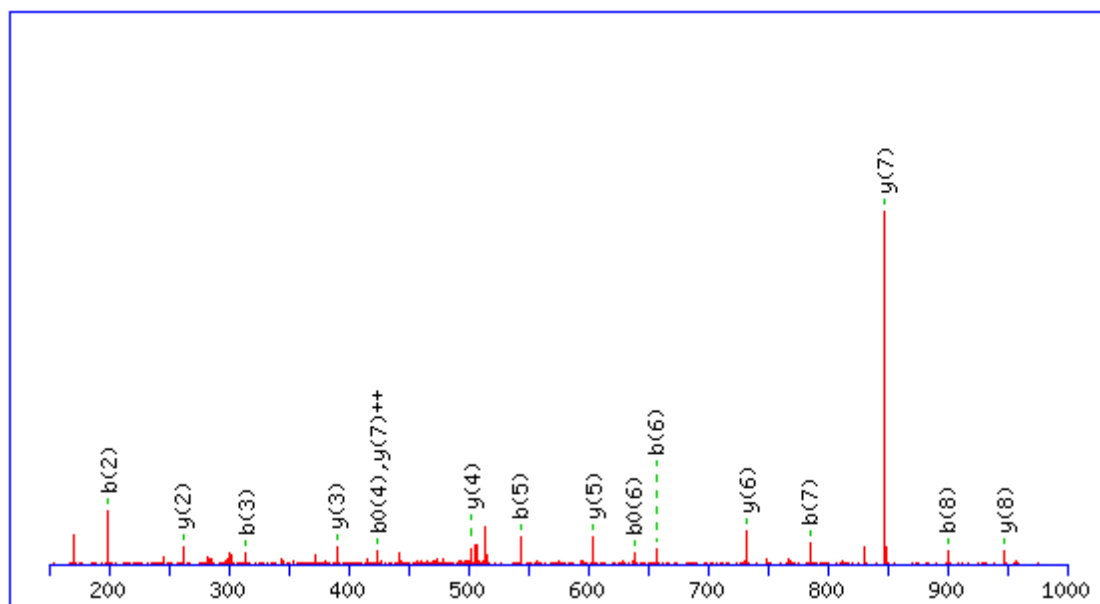
Monoisotopic mass of neutral peptide Mr(calc): 1045.529144

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N3 : Deamidated (NQ)

Ions Score: 64 Expect: 0.0027



MS/MS Fragmentation of **TTGGCNNPCTVFKTDEYCCNSGSCnATDYSR**

Found in **gi|7406714**, putative thaumatin-like protein [Vitis vinifera]

Monoisotopic mass of neutral peptide Mr(calc): 3598.332367

Fixed modifications: Carbamidomethyl (C)

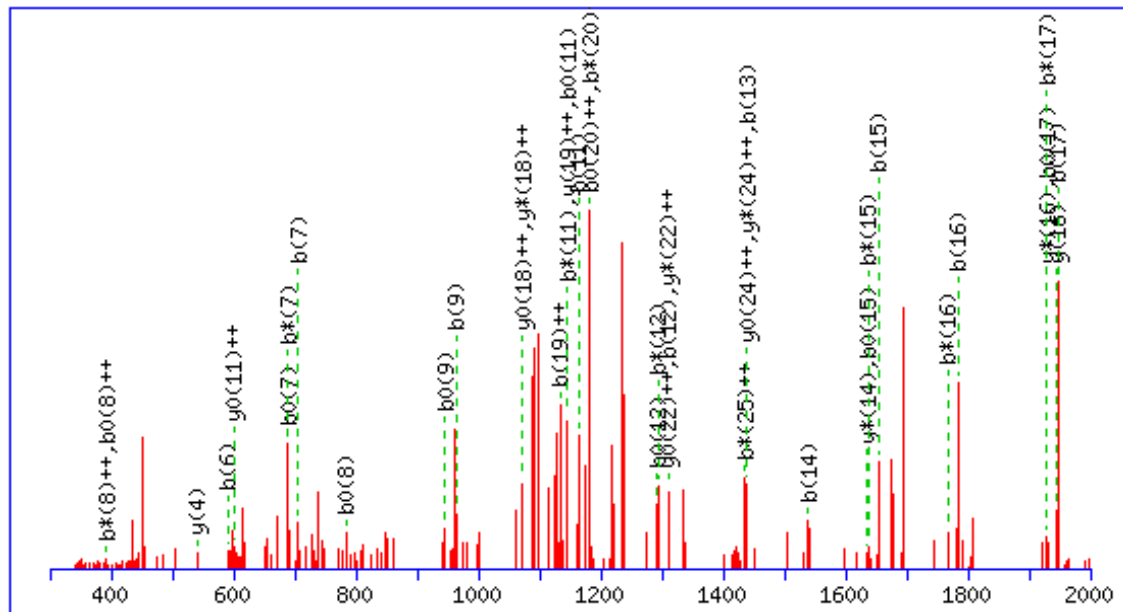
Variable modifications:

N7 : Deamidated (NQ)

N20 : Deamidated (NQ)

N25 : Deamidated (NQ)

Ions Score: 19 **Expect:** 2.9e+02



MS/MS Fragmentation of **DYCSQLGVSPGDnLTC**

Found in **gi|2306813**, class IV endochitinase [Vitis vinifera]

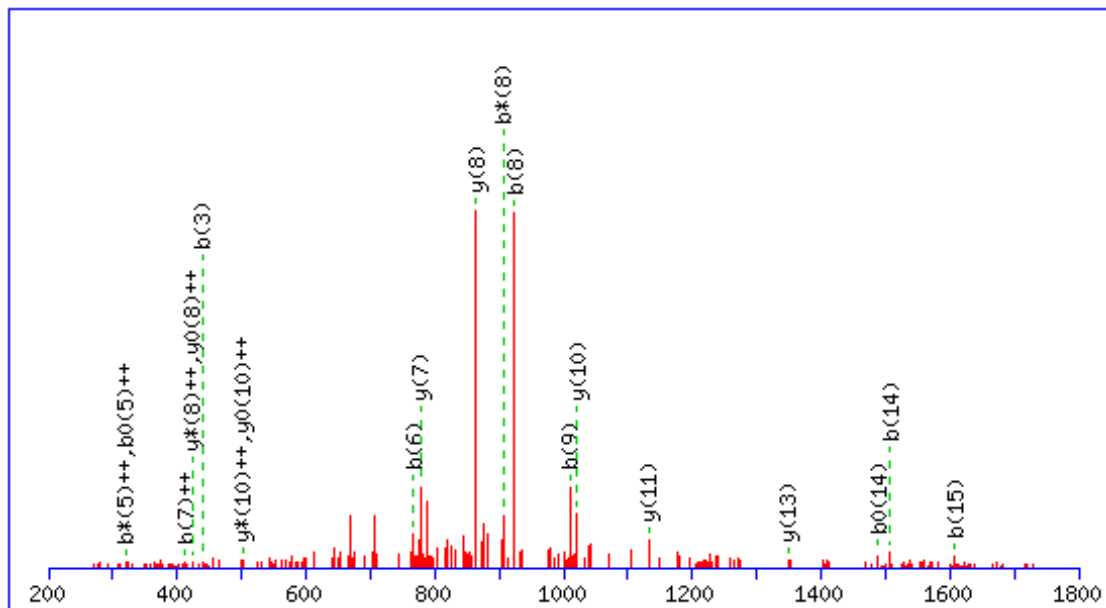
Monoisotopic mass of neutral peptide Mr(calc): 1785.718582

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N13 : Deamidated (NQ)

Ions Score: 21 **Expect:** 1.1e+02



MS/MS Fragmentation of **SCSCPTGCNSDDKCPCGnKT**

Found in [gi|6321844](#), Metallothionein, binds copper and mediates resistance to high concentrations of copper and cadmium; locus is variably amplified in different strains, with two copies, CUP1-1 and CUP1-2, in the genomic sequence reference strain S288C; Cup1-1p [Saccharomyces

Monoisotopic mass of neutral peptide Mr(calc): 2204.753738

Fixed modifications: Carbamidomethyl (C)

Variable modifications:

N9 : Deamidated (NQ)

N18 : Deamidated (NQ)

Ions Score: 38 **Expect:** 1.5

