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Evaluation of Microwave-Accelerated Residue-Specific Acid Cleavage for Proteomic Applications

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Microwave-accelerated proteolysis using acetic acid has been shown to occur specifically on either or both sides of aspartic acid residues. This chemical cleavage has been applied to ovalbumin and several model peptides to test the effect on some of the more common post-translational modifications. No oxidation of methionine or cysteine was observed; however, hydrolysis of phosphate groups proceeds at a detectable rate. Acid cleavage was also extended to the yeast ribosome model proteome, where it provided information on 74% of that proteome. Aspartic acid occurs across the proteome with approximately half the frequency of the combined occurrence of the trypsin residues lysine and arginine, and implications of this are considered.

Keywords: proteolysis • long peptides • acid cleavage • microwave acceleration • ribosomes • post-translational modifications • aspartic acid • Orbitrap

Introduction

We have previously reported the use of microwave-accelerated acid cleavage to denature *Bacillus* spores and MS-2 bacteriophage and to form peptides from their solubilized proteins in a single step. Protein cleavage was observed to occur specifically on either side of aspartic acid[1–3] and may occur on both sides, resulting in removal of aspartic acid from the chain.

This proteolytic chemical reaction was reported as early as 1967,[4] when kinetic studies demonstrated that acid-catalyzed cleavage occurs at least 100 times faster at aspartic acid than any other residues. The mechanisms shown in Figure 1, adapted from Li et al.,[5] feature formation of a five-membered ring leading to cleavage of the polypeptide backbone on the C-terminal side and a six-membered intermediate, which can open with cleavage of the amide bond on the N-terminal side of aspartate. Preferential cyclization and subsequent ring opening by water require a pH of <2.1, and catalysis is reported to be independent of the acid used.[5,6]

Proteolysis has been observed to be significantly accelerated at temperatures exceeding 108 °C.[6] Conventional thermal sources have mainly been used for the reaction to date; however, recently, several laboratories have reported that microwave technology accelerates cleavage significantly.[1,7–9] The studies reported here aim to evaluate the suitability of the reaction and its peptide products for use in proteomic strategies. Observations of the effect of hot acid processing on a selection of the functional groups that are encountered as post-translational modifications of proteins are reported, and a model proteome is processed and analyzed to evaluate the potential for larger scale applications.

Experimental Section

Analysis of Ovalbumin and Peptide Standards. Hen ovalbumin, calcitonin, and YGGFMRF were obtained from Sigma (St. Louis, MO). The phosphopeptide TRDIYETDYphosYRK was obtained from Biomol International (Plymouth, PA). Solutions were prepared at 0.1 mg/mL in Milli-Q grade water. Microwave-accelerated acid hydrolysis was carried out in a Discover Benchmate microwave system (CEM Corp., Matthews, NC) equipped with a 45 mL digestion vessel and a fiber optic temperature probe. A 43.75 μL aliquot of the protein/peptide solution was acidified to 12.5% with 6.25 μL of glacial acetic acid (Fisher Scientific, Pittsburgh, PA) in a 300 μL glass sample holder and placed in the digestion vessel. All digestions were carried out at a constant temperature of 140 ± 5 °C and 300 W for 0.5−20 min. Fifteen seconds was added to the reported digestion times to allow temperatures to exceed 108 °C. MALDI analysis was carried out with a 337 nm nitrogen laser on a Shimadzu (Columbia, MD) Axima CFR Plus time-of-flight mass spectrometer. Peptide mass spectra were acquired in linear mode, using α-cyanohydroxycinnamic acid (CHCA) or sinapinic

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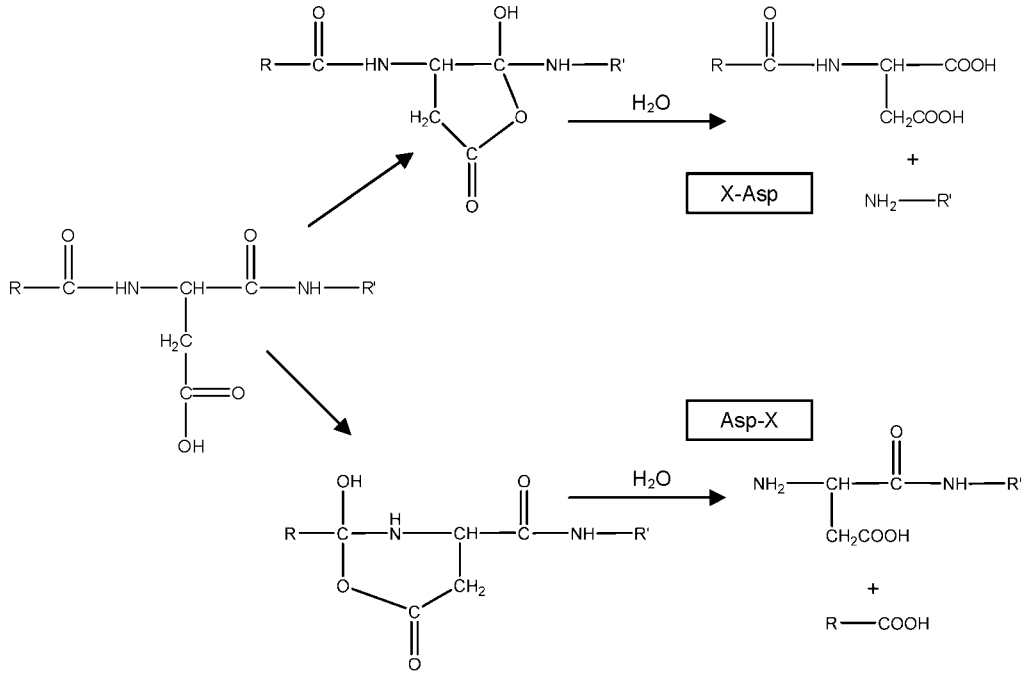

Figure 1. Reaction mechanisms proposed for acid-catalyzed proteolysis at pH <2 and >105 °C (adapted from ref 5).

acid, as averages of 200 profile scans. Although this laboratory has evaluated a range of acids,[1] all the work reported here was carried out with 12% acetic acid.[2] The processing time for each protein or mixture was determined empirically, by examining the products with MALDI mass spectrometry to optimize peptide diversity and signal abundance.

Analysis of the Yeast Ribosomal Proteome. A 10 μL suspension of intact ribosomes (~16 pmol) isolated from *Saccharomyces cerevisiae* cells[10] was acidified by addition of 100 μL of glacial acetic acid. Precipitated RNA was pelleted during 10 min of centrifugation (14000 rpm). The supernatant containing the ribosomal proteins was collected and adjusted to 12.5% acetic acid by addition of 600 μL of Milli-Q grade water. A 100 μL aliquot was transferred to a 300 μL glass sample holder and subjected to microwave heating in a laboratory microwave system equipped with a fiber optic temperature probe. Digestions were carried out at 140 ± 5 °C for 20 min. Once the digested sample had cooled, 0.3 μL of it was applied to a MALDI sample plate and allowed to air-dry; 0.3 μL of a CHCA matrix solution [10 mg/mL in a 70% (v/v) acetonitrile/30% (v/v) deionized water mixture containing 0.1% trifluoroacetic acid] was then added to the sample plate and analyzed by MALDI-TOF as described above.

For automated analysis using electrospray, the peptides were separated on a C-18 column (50 cm) (MicroTech Inc., Vista, CA) interfaced through an electrospray source to an LTQ-Orbitrap (Thermo Electron, San Jose, CA). A 30 to 60% acetonitrile gradient was developed over 130 min. Precursor masses were determined using the Orbitrap, while the LTQ was programmed to select the five most abundant ions in each scan for CID and MS/MS analysis. Ions were scanned between m/z 200 and 4000. Selected ions were excluded for 30 min. This analysis was carried out in the Marlene and Stewart Greenebaum Cancer Center Proteomics Core Facility.

To identify peptide products and the proteins from which the peptides are derived, spectra from the MS/MS experiments were converted to MGF format and subjected to MASCOT searches (Matrix Science Ltd., London, U.K.). Mass tolerances were ±0.05 and ±0.8 Da for precursor and fragment ions, respectively. A searchable yeast ribosomal protein database was constructed from the online Ribosomal Protein Gene Database[11] to facilitate analysis of ribosomal peptides. To accomplish these searches, the "enzyme" designation in the MASCOT search program was configured to accommodate cleavage on either side of aspartate.

Results and Discussion

Microwave-accelerated acid hydrolysis was applied to standard peptides and to a standard protein, and the products were examined. Ovalbumin is a 385-amino acid glycoprotein with a calculated molecular mass of 42750 Da based only on its amino acid sequence. In addition to glycosylation at Asn-292, ovalbumin is known to be acetylated at Gly-1 and to hold two potential phosphorylation sites, Ser-69 and Ser-345.[12] These post-translational modifications make ovalbumin a good model protein for evaluation of the new proteolytic method. Figure 2 presents a MALDI spectrum of the peptide products recovered from a 5 min microwave-accelerated acid cleavage of ovalbumin. A number of peptides are detected, and many of the peaks occur in pairs separated by 115 Da. These are peptides with and without a terminal aspartic acid, the presence of which indicates that acid cleavage is occurring at D residues, as expected.

These peptide masses were automatically searched against the hen ovalbumin sequence and assigned as fragments of hen ovalbumin cleaved at D. The list of peptides automatically identified by MASCOT is provided in Table 1. A number of peptides are identified that carry the acetyl group on the N-terminal glycine. Thus, we conclude that acetylation is preserved under the conditions used here for acid cleavage. In addition, no artifactual acetylation was observed. The pair of internal peptides, with m/z values of 3024.3 and 3139.6, is assigned as [140–166] and [140–167], in which N-terminal glutamines have been converted to pyroglutamates by the hot acid treatment. This is expected to occur when the amino

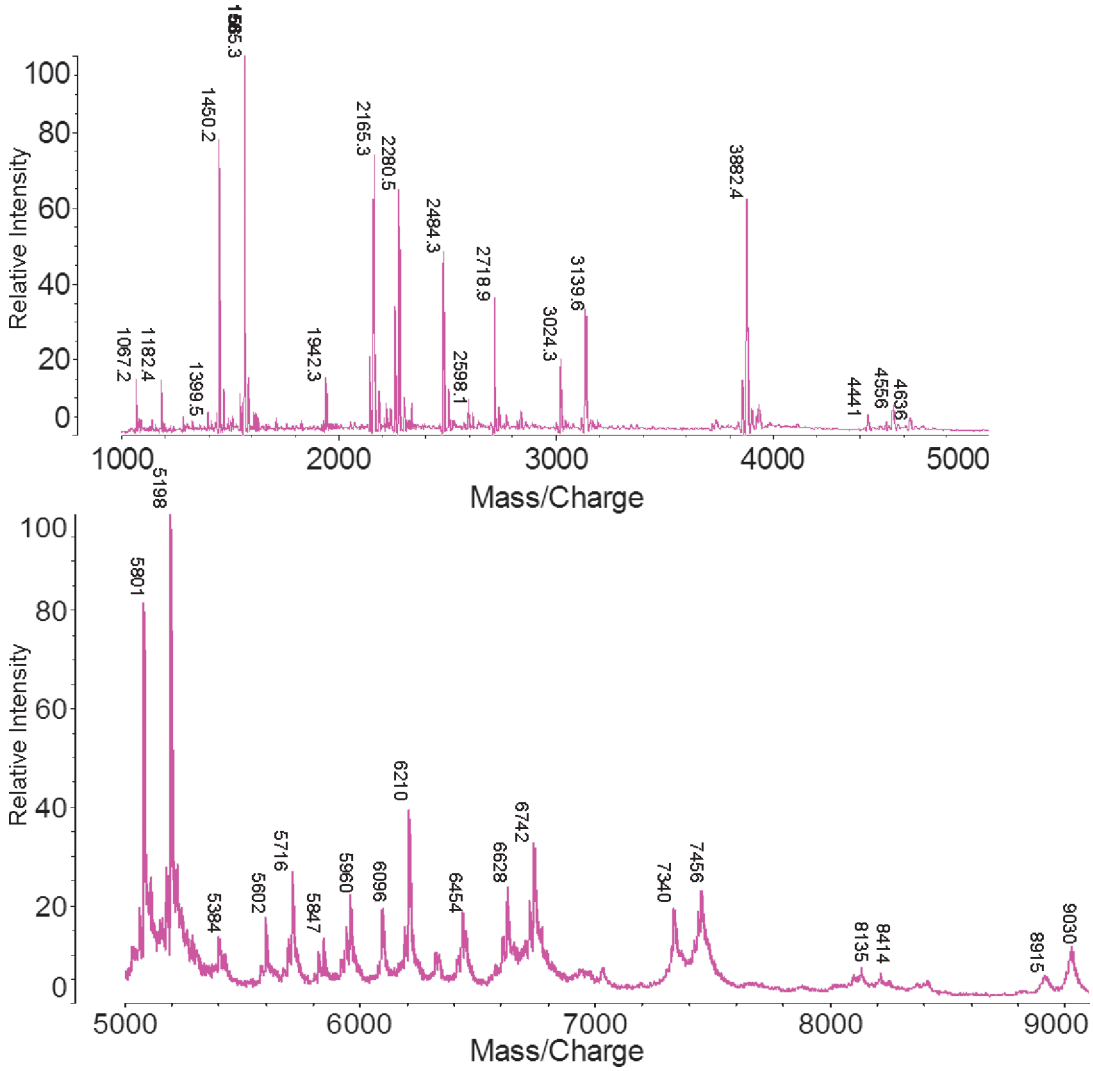

Figure 2. MALDI survey spectrum of ovalbumin digestion products, showing pairs of peptides with and without aspartic acid. The top panel was measured with CHCA matrix. The bottom panel was measured with sinapinic acid matrix.

terminus of a new peptide product comprises glutamine. MASCOT and most search programs are constructed to accommodate this cyclization in identifying sequences and peptides.

Molecular ions of the peptide pair [305–350] and [305–351], which contain the phosphorylation site Ser-345, are shown in Figure 3. The *m*/*z* values indicate the presence of a pair of phosphopeptides and their dephosphorylated analogues. Since the extent of phosphorylation of the commercial protein was unknown, no conclusion could be made about the stability of the phosphate ester bond. The phosphopeptide TRDIYETDYphosYRK was used to examine the possibility of hydrolysis. Complete phosphorylation of the peptide was confirmed by MALDI-TOF before hot acid treatment. Spectra of products from 45 s and 5 min digestions are shown in Figure 4, which indicate that hydrolysis of the phosphate group occurs in this case, in the same time frame as proteolysis.

The effects of hot acid processing on the sulfur-containing residues methionine and cysteine were studied using YGGFMRF and the cyclic disulfide peptide calcitonin. Before and after spectra of YGGFMRF revealed no increase in the amount of oxidized methionine present. Similarly, spectra of calcitonin showed no oxidation of either Met or Cys. As expected, no reduction of the disulfide bond was detected.

The list of peptides from ovalbumin (Table 1) shows overlapping peptides that cover 84% of the protein sequence. The single missing polypeptide is [248–304], which carries the carbohydrate side chain. This absence is probably the result of suppression of the heavy heterogeneous glycopeptide in the MALDI process. The fate of the ovalbumin carbohydrate side chain in the acid reaction is unknown; however, a recent report on microwave-assisted acid hydrolysis of carbohydrates indicates that glycolysis takes place nonspecifically, on a time scale comparable to that of proteolysis.[13]

To evaluate the suitability of this method for high-throughput proteomic workflows, the ribosomal proteome from *S. cerevisiae* was studied. The small and large subunits of the yeast ribosome comprise 78 proteins, in essentially equimolar amounts. These RNA-binding proteins are characterized by a high density of the basic residues Lys and Arg. To set benchmarks for successful cleavage and characterization, the model proteome was digested *in silico*. Asp-specific digestion produces 387 peptides in the *m*/*z* range of 500–5000, the range expected to be most readily analyzed by electrospray and tandem mass spectrometry. Tryptic digestion at Arg and Lys, with no missed cleavages, would be expected to produce 1040 peptides in that same mass range. The difference in the frequency of cleavages produces different

Table 1. List of Experimentally Observed Asp-Specific Peptides from Ovalbumin, As Assigned by MASCOT[a]

[M+H]+ calc.	[M+H]+ obs.	AA Position	Amino Acid Sequence
1182.2	1182.4	350-360, 351-361	(D)AASVSEEFRA(D)
1067.2	1067.2	351-360	AASVSEEFRA
1335.4	1399.5** +Na	1-13	GSIGAASMEFCFD
1564.8	1565.3	47-59, 48-60	(D)STRTQINKVVRF(D)
1449.8	1450.2	48-59	STRTQINKVVRF
1861.9	1942.3*	336-356	AGREVVGSAEAGVDAASVSEE
2279.6	2280.5	47-66,48-67	(D)STRTQINKVVRFDKLPGFG(D)
2164.9	2165.3	48-66	STRTQINKVVRFDKLPGFG
2597.1	2598.1	167-189, 168-190	(D)SQTAMVLVNAIVFKGLWEK AFK(D)
2482.1	2484.3	168-189	SQTAMVLVNAIVFKGLWEKAFK
2719.4	2718.9	362-385	HPFLFCIKHIATNAVLFFG RCVSP
3155.5	3139.6	139-166	pyro-EARELINSWVESQTNGIIR NVLQPSSVD
3040.5	3024.3	139-165	pyro-EARELINSWVESQTNGIIR NVLQPSSV
3882.6	3882.4	351-385	AASVSEEFRADHPFLFCIK HIATNAVLFFGRCVSP
4555	4556, 4636*	304-349, 305-350	(D)VFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGV(D)
4440	4441	305-349	VFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGV
5155	5198**	1-47	GSIGAASMEFCFDVFKELKV HHANENIFYCPIAIMSALAM VYLGAKD
5040	5082**	1-46	GSIGAASMEFCFDVFKELKV HHANENIFYCPIAIMSALAM VYLGAK
5384.3	5384	13-59, 14-60	(D)VFKELKVHHANENIFYCPI AIMSALAMVYLGAKDSTRTQINKVVRF(D)
5718.1	5716	304-360, 305-361	(D)VFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGVDAASVSEEFRA(D)
5603.1	5602	305-360	VFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGVDAASVSEEFRA
5848.6	5847	138-190	DQARELINSWVESQTNGIIR NVLQPSSVDSQTAMVLVNAIVFKGLWEKAFKD
6454	6454	190-246	DEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMSMLVLLP
6210	6210	192-246, 193-247	(D)TQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMSMLVLLP(D)
6095	6095	193-246	TQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMSMLVLLP
6701	6742**	1-60	GSIGAASMEFCFDVFKELKV HHANENIFYCPIAIMSALAM VYLGAKDSTRTQINKVVRFD
6586	6628**	1-59	GSIGAASMEFCFDVFKELKV HHANENIFYCPIAIMSALAM VYLGAKDSTRTQINKVVRF
7416	7456**	1-67	GSIGAASMEFCFDVFKELKV HHANENIFYCPIAIMSALAM VYLGAKDSTRTQINKVVRFD KLPGFGD
7301	7341**	1-66	GSIGAASMEFCFDVFKELKV HHANENIFYCPIAIMSALAM VYLGAKDSTRTQINKVVRFD KLPGFG
8137	8135	96-166, 97-167	(D)VYSFSLASRLYAEERYPILPEYLQCVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSV(D)
8138	8217*	67-137, 68-138	(D)SIEAQCGTSVNVHSSLRDI LNQITKPNDVYSFSLASRLY AEERYPILPEYLQCVKELYR GGLEPINFQTAA(D)
8417	8414	304-385	(D)VFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGVDAASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP
9033	9030	167-246, 168-247	(D)SQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMSMLVLLP(D)
8917	8915	168-246	SQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMSMLVLLP

[a] One asterisk indicates phosphorylation, and two asterisks indicate acetylation.

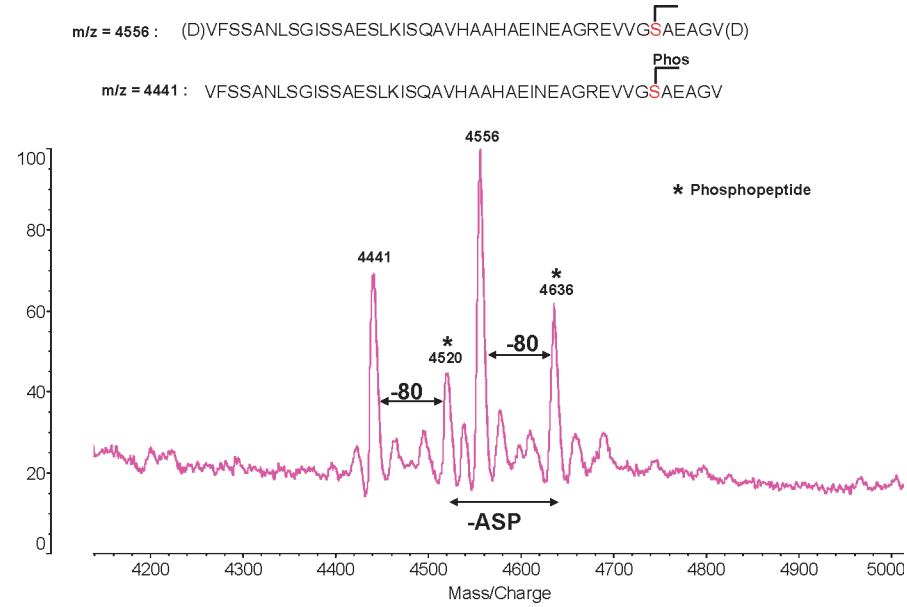

Figure 3. Expanded MALDI spectrum of a partially phosphorylated pair of peptides.

distributions of peptide lengths, as shown in Figure 5. More than 80% of the tryptic peptides contain fewer than 10 residues. We expect that the more heterogeneous mixture of longer peptides would be more readily fractionated by

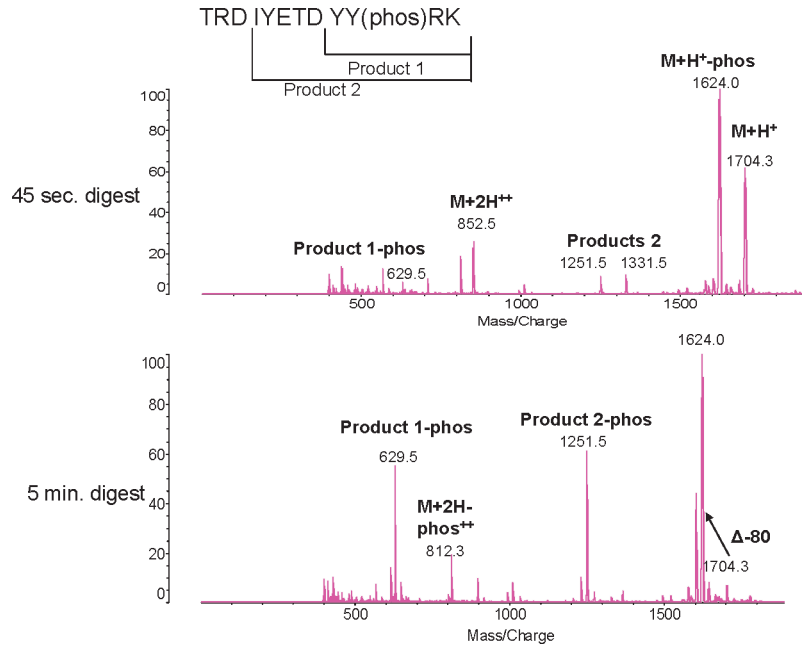

Figure 4. MALDI spectra of a phosphopeptide following acid cleavage for 45 s and 5 min.

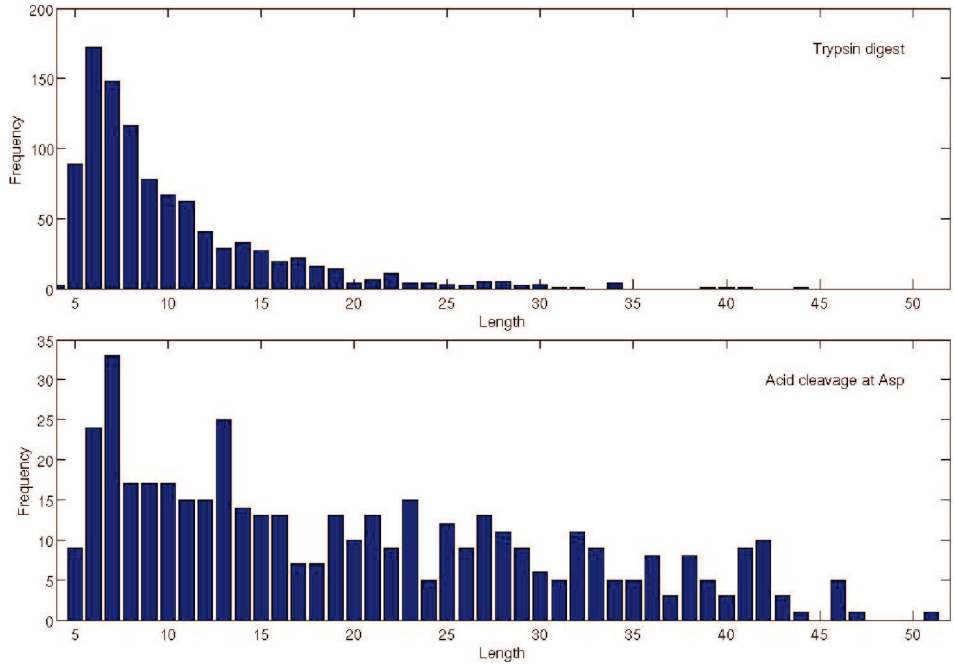

Figure 5. Number of residues predicted for yeast ribosomal peptides produced by (top) trypsin digestion and (bottom) Asp-specific acid cleavage.

HPLC.[14] There are also implications for coverage of the yeast ribosomal proteome. Figure 6 summarizes the coverage of each of the 78 ribosomal proteins by peptides in the m/z range 500−5000. In the case of tryptic digestion, blue or unanalyzed regions generally result from production of peptides with masses of <500 Da. In the case of acid cleavage at Asp, blue regions generally reflect production of peptides with masses of >5000 Da.

In addition, the peptide products have very different distributions of basic residues, as shown in Figure 7. Lys and Arg are the most likely sites for protonation[15,16] by electrospray, and their number is expected to influence the charge state, m/z values, and accessibility of peptides for analysis. Tryptic peptides can be seen in Figure 7 to carry one basic residue (at the C-terminus), with the exception of the peptides that terminate each protein and those that contain Arg or Lys followed by Pro. (The latter are considered not to be cut by trypsin.[17]) Interestingly, among the peptides predicted to be produced by cleavage at Asp, the dominant species also carry a single Lys or Arg. However, a significant number are expected to contain no basic residue, and the distribution of basic charge centers extends beyond 10 in the mass range examined in Figure 7. In addition, few products of residue-specific acid cleavage are expected to end in K or R. Thus, the characteristics of the set of peptides expected from cleavage on either side of aspartic acid differ from those produced by the widely used

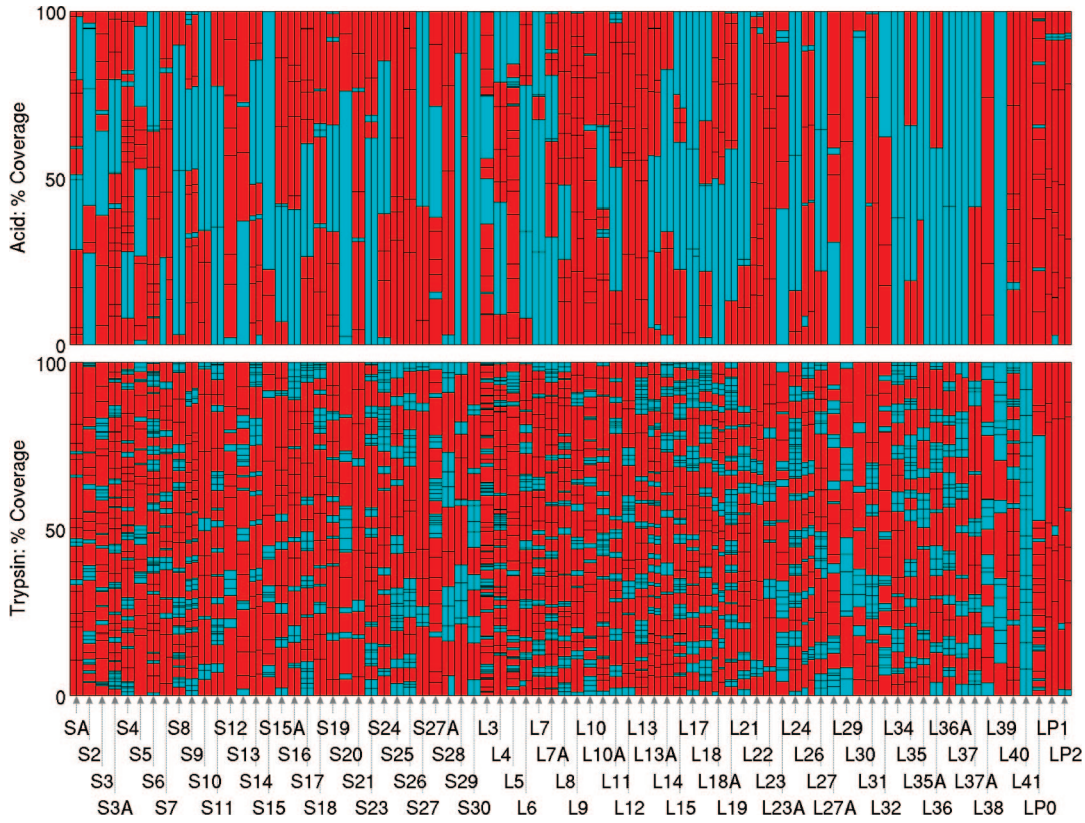

Figure 6. Predicted percent coverage of the 78 yeast ribosomal proteins by 500–5000 Da peptides. Each ribosomal protein is displayed individually. Red indicates the extent of protein sequence covered by each peptide. Teal areas are not covered. Top: products of acid cleavage. Bottom: products of trypsin cleavage.

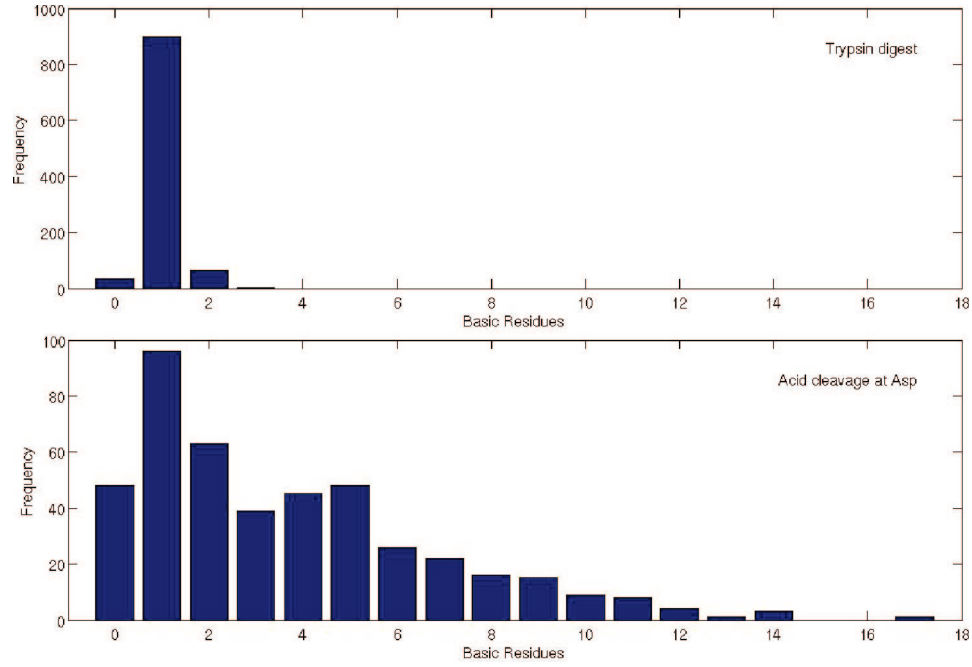

Figure 7. Number of basic residues (lysine and arginine) predicted for yeast ribosomal peptides produced by (top) trypsin digestion and (bottom) Asp-specific acid cleavage.

tryptic digestion, and it was apparent that the suitability of this novel peptidome needed to be tested experimentally in a high-throughput workflow.

Microwave conditions for the yeast ribosome were optimized on the basis of MALDI analysis of the unfractionated product mixture. As in Figure 2, many new peaks, including pairs of signals 115 Da apart, indicated that cleavage was occurring. Finally, a 20 min processing time was chosen for the ribosomal proteins (see Experimental Section). Fractionation of the product mixture by HPLC and analysis by collisional dissociation on an LTQ ion trap, with precursor masses measured in high resolution by the interfaced Orbitrap, led to the identifica-

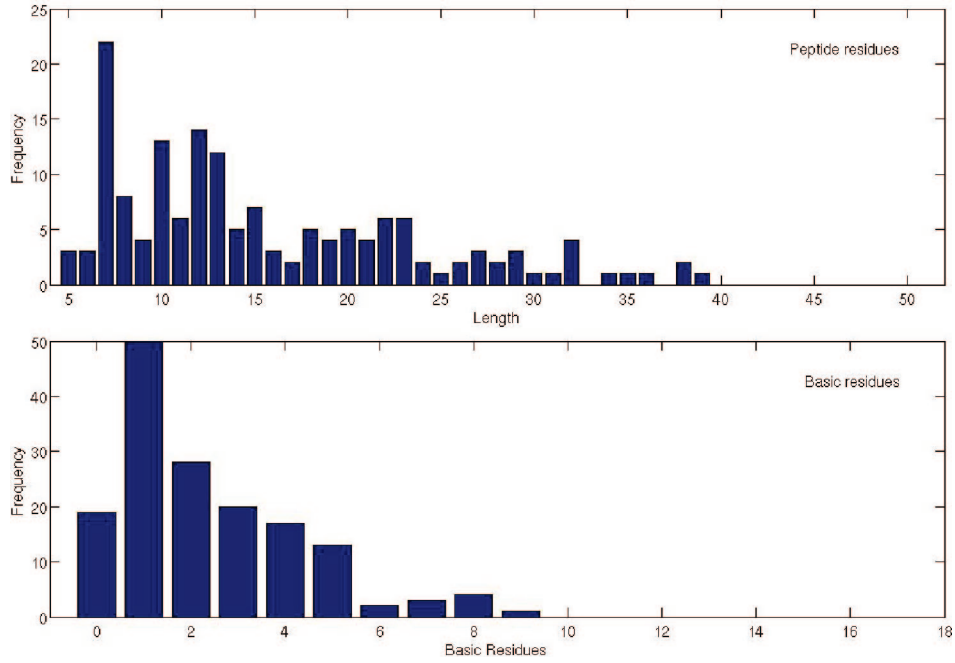

Figure 8. Number of residues (top) and number of basic residues (bottom) present in peptides experimentally identified after aspartate-specific acid cleavage of yeast ribosome proteins.

tion of 247 peptides with MASCOT *E* values of ≤0.05 (see Table 1 of the Supporting Information). Peptide identification specificity was verified using a reverse database search to estimate the false discovery rate (FDR) at 7%. Ninety of the peptides identified were peptide pairs (with and without terminal Asp); thus, the number of distinct peptides identified was 157. These peptides enabled identification of 58 of the 78 ribosomal proteins (see Table 1 of the Supporting Information). It is expected that coverage of this proteome can be increased by repetitive LC−MS/MS analyses,[18] and/or the use of isoelectrofocusing[19] or another orthogonal peptide separation ahead of reverse phase HPLC.

In an independent evaluation of the specificity of the process, all the electrospray spectra were searched a second time, with "no enzyme specificity" designated. A total of 29 peptides were identified with Expect values of $\leq 10^{-5}$. Seventeen percent of these (five) were observed with a cleavage other than Asp at one end, and none contained incompatible cleavages at both ends. One hundred ninety-two peptides were identified with Expect values of <0.05 (including the 29 previously described peptides). Twenty-five percent (49) of these were observed with cleavage other than D at one end. Five percent (nine) contained incompatible cleavages at both ends. The remaining peptides were formed by D cleavages at both ends and were essentially convergent with peptides identified by searching with D cleavages required (see Tables 1 and 2 of the Supporting Information). Among the 67 variant termini (in 58 total variant peptides) identified in this search, 37 are associated with Glu, 15 with Asn, and 11 with Pro. The occurrence of these cleavages, relative to cleavage at Asp, is expected to depend on the length and pH of the incubation.[3–6]

Plots of the distributions of length and the number of basic residues in the 247 peptides formed by Asp cleavage are shown in Figure 8. The observed distributions in Figure 8 may be compared to the predicted distributions in Figures 5 and 7. We conclude that there is no evidence of a bias in the set of identified peptides related to the number of basic residues, once we account for the confounding factors of molecular weight and peptide length. Interestingly, there is no statistical evidence that peptides with neither K nor R are under or over-represented in the set of identified peptides with at most 25 residues. There does appear to be a significant bias against characterization of peptides of 25 residues or more, and a significant bias in favor of characterization of peptides of 10–15 amino acids. Peptide length is correlated with molecular weight, and it seems most reasonable to assign the bias to molecular weight. It is likely that the under-represented peptides reflect the limitations of multiple charging,[22] collisional activation, and database search engines applied to longer peptides, rather than something unique to the products of acid hydrolysis.

Among the 247 ribosomal peptides formed exclusively by cleavages at D, 89 were found to carry D on the carboxyl terminus, seven peptides had D at their amino terminus, 150 peptides contained no D, and one peptide was identified that carried two D residues, one on each end (Table 1 of the Supporting Information). On the basis of this count and other observations, the initial cleavage is proposed to occur preferentially on the C-terminal side of aspartate residues. With reference to Figure 1, this indicates that the path that includes the five-membered cyclic intermediate is the most efficient. This is consistent with early studies by Bruice and Pandit,[20] who studied the formation of cyclic anhydrides from succinate monoesters and glutarate monoesters. They report that five-membered cyclic anhydrides are formed 230 times faster than six-membered rings, although both kinds of anhydrides have similar rates of solvolysis.

Conclusions

This study and earlier[1–3] studies with standard proteins, microorganisms, and the yeast ribosome proteome confirm that acid-catalyzed proteolysis carried out in a microwave oven below pH 2 and at temperatures around 140 °C occurs with high selectivity at aspartic acid residues. The use of the

microwave[21] allows the use of short reaction times, in the 0.5−20 min range. The possibility of cleavage on either or both sides of aspartic acid must be accommodated in manual or automatic interpretation of MS/MS spectra, and we have modified the enzyme rule for MASCOT to model cleavage on either side of Asp.[22] This modification has recently been implemented by Matrix Science under the "formic acid" button, in order to provide public access.

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Supporting Information Available: Identified proteins and peptides from the *S. cerevisiae* ribosome identified by searching with the requirement that cleavage occur at D (Table 1) and identified proteins and peptides from the *S. cerevisiae* ribosome identified by searching with no specific cleavage (Table 2). This information is available free of charge via the Internet at http://pubs.acs.org.

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