

TECHNICAL MANUAL

rChymoSelect™ MS Grade Protease

Instructions for Use of Products
VB8100 and VB8200

rChymoSelect™ MS Grade Protease

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1. Description

The rChymoSelect™ MS Grade Protease^(a) is a novel recombinant chymotryptic protease developed for peptide mapping analysis of biotherapeutic proteins and bottom-up proteomics workflows. The protease was engineered to address common limitations associated with traditional bovine pancreas-derived chymotrypsins, including extensive nonspecific cleavages and autoproteolysis.

Unlike the traditional bovine-derived chymotrypsins, rChymoSelect™ MS Grade Protease belongs to a distinct class of chymotryptic proteases characterized by enhanced cleavage specificity and intrinsic resistance to autoproteolysis (Figure 1, Panel A and Figure 2, Panel A). The protease is produced in microbial culture under controlled manufacturing conditions and extensively purified to support high enzymatic reproducibility and purity. In contrast to traditional chymotrypsins, rChymoSelect™ MS Grade Protease does not require calcium for structural or enzymatic stability.

rChymoSelect™ MS Grade Protease preferentially cleaves after tyrosine (Y), phenylalanine (F) and leucine (L) residues, with occasional cleavage observed after selected methionine (M) residues, while significantly reducing nonspecific cleavage after histidine (H), threonine (T), valine (V), serine (S) and other residues commonly observed with the traditional chymotrypsins (Figure 1, Panel B and Figure 2, Panel B). Cleavage activity at tryptophan (W) residues is selectively inhibited, making the protease suitable for characterization of tryptophan modifications. Cleavage efficiency at leucine sites is influenced by local sequence context, digestion time and protease-to-substrate (also referred to as the enzyme-to-substrate, E:S) ratio (Table 1). Cleavage is also reduced when proline (P) directly follows tyrosine (Y) or phenylalanine (F).

Recommended digestion conditions include 0.8M guanidine hydrochloride (GuHCl) in 50mM HEPES or Tris-HCl buffer (pH 7.5), an enzyme-to-substrate ratio of 1:20 to 1:40 and incubation for 2 hours at 25°C. Under these mildly denaturing conditions, cleavage site accessibility is enhanced while maintaining high cleavage specificity. For MS/MS data analysis, we recommend allowing 2–3 missed cleavages to improve peptide identification containing leucine missed cleavages.

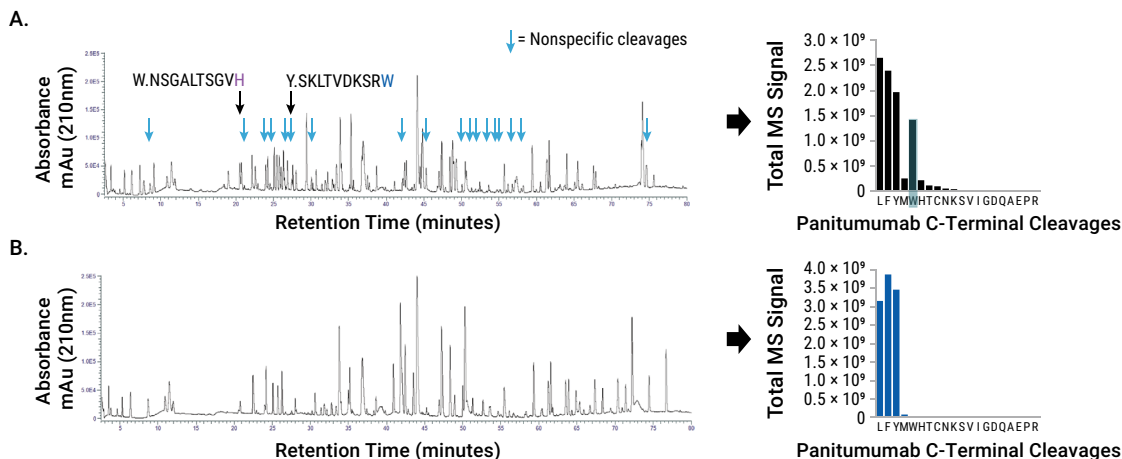


Figure 1. Enhanced cleavage specificity with rChymoSelect™ MS Grade Protease. Panitumumab digests generated with traditional chymotrypsin (**Panel A**) or rChymoSelect™ MS Grade Protease (**Panel B**) were analyzed by UV-UPLC. Digestion conditions were 50mM Tris-HCl (pH 7.5), 0.8M GuHCl, E:S = 1:40, 25°C and 2 hours. Traditional chymotrypsin digestion additionally included 10mM CaCl₂. Representative cleavage sites at W and H residues are labeled in **Panel A** (black arrows). Total extracted ion chromatogram (XIC) intensity grouped by cleavage-site amino acid residue for traditional chymotrypsin and rChymoSelect™ MS Grade Protease. Traditional chymotrypsin chromatograms contain multiple nonspecific peptide peaks (blue arrows). In contrast, rChymoSelect™ MS Grade Protease chromatograms show peptide peaks corresponding to specific cleavages. The superior cleavage specificity of rChymoSelect™ MS Grade Protease is also reflected by the analysis of C-terminal cleavages where C-terminal cleavages are demonstrated to occur predominantly after Y/F/L.

1. Description (continued)

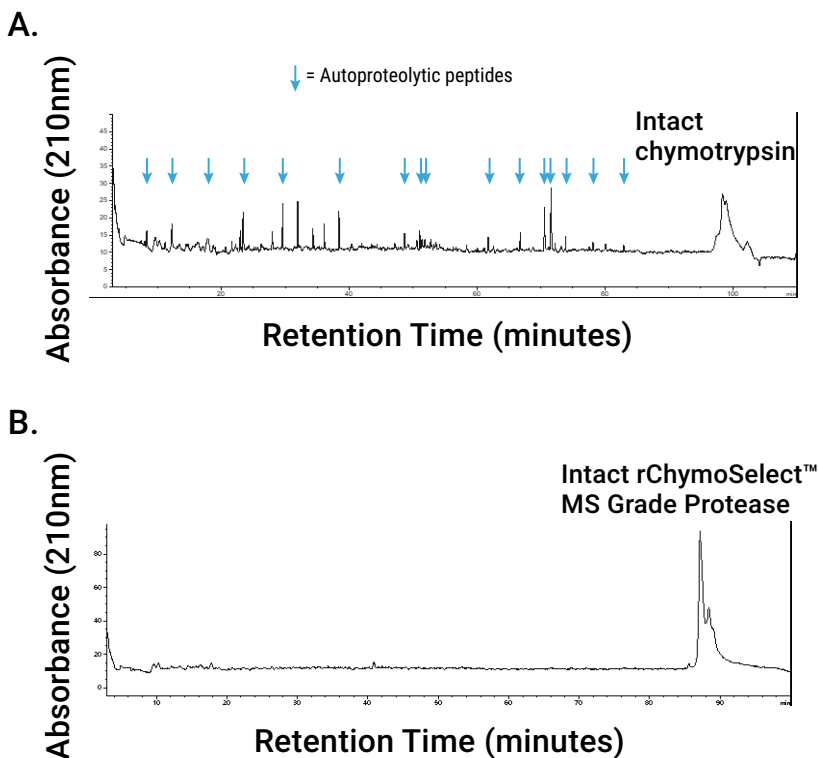


Figure 2. Autoproteolytic resistance with rChymoSelect™ MS Grade Protease. Traditional chymotrypsin (**Panel A**) and rChymoSelect™ MS Grade Protease (**Panel B**) were incubated under digestion conditions without substrate and analyzed by UV-UPLC. Traditional chymotrypsin chromatograms contain multiple autoproteolytic peptide peaks (blue arrows). In contrast, rChymoSelect™ MS Grade Protease chromatograms show a predominant peak corresponding to the intact protease with negligible autoproteolytic peptide formation.

Table 1. rChymoSelect™ MS Grade Protease Digestion Efficiency Across Enzyme-to-Substrate Ratios. Digestions were performed at E:S ratios of 1:100, 1:40, 1:20 and 1:10. The table reports: (i) the percentage of identified peptides terminating at the expected rChymoSelect™ cleavage residues (Y/F/L/M); (ii) percent nonspecific cleavage calculated from total extracted ion chromatogram (XIC) intensity; (iii) residue-specific cleavage efficiency, defined as the XIC intensity of fully cleaved peptides at a given residue divided by the total XIC intensity of all peptides containing that residue; and (iv) average peptide length. Values represent the average of three independent digestions (n = 3).

Enzyme-to-Substrate Ratio (E:S)	1:100	1:40	1:20	1:10
Percent Peptide with C-terminal cleavages at Y/F/L/M	99.9%	99.8%	99.4%	98.5%
Percent Nonspecific Cleavages	0.1%	0.2%	0.6%	1.5%
Percent fully cleaved Y (Primary cleavage site)	84%	86%	87%	91%
Percent fully cleaved F (Primary cleavage site)	52%	54%	54%	53%
Percent fully cleaved L (Secondary cleavage site)	17%	26%	31%	38%
Percent fully cleaved M (Secondary cleavage site)	10%	14%	17%	32%
Average peptide length (amino acid number)	17	15	14	13

rChymoSelect™ maintains highly specific cleavage across a broad range of digestion conditions.

More than 98.5% of peptides terminated at the expected Y/F/L/M cleavage sites at all E:S ratios, while non-specific cleavage remained low ($\leq 1.5\%$). Cleavage at the primary specificity residues, tyrosine (Y) and phenylalanine (F), reached a plateau across all conditions (Y: 84–91%; F: 52–54%), indicating that these sites are no longer enzyme-limited even at the lowest E:S ratio. This demonstrates robust and reproducible primary-site cleavage regardless of enzyme loadings.

In contrast, cleavage at the secondary specificity residues, leucine (L) and methionine (M), increased with enzyme amount. The proportion of fully cleaved L-containing peptides increased from 17% to 38%, while fully cleaved M-containing peptides increased from 10% to 32% as the E:S ratio increased from 1:100 to 1:10. The increased cleavage at secondary sites reduced the average peptide length from 17 to 13 amino acids.

These results demonstrate that primary-site cleavage is largely insensitive to enzyme amount under the tested conditions, whereas secondary-site cleavage provides a tunable parameter for controlling peptide size. An E:S ratio of 1:20 provides an effective balance between efficient primary-site cleavage and controlled secondary-site cleavages, making it the recommended condition for routine LC–MS peptide mapping.

2. Product Components and Storage Conditions

2.A. Product Components

PRODUCT	SIZE	CAT. #
rChymoSelect™ MS Grade Protease	25µg	VB8100
rChymoSelect™ MS Grade Protease	100µg	VB8200

2.B. Product Storage Conditions

Upon receipt, store the lyophilized rChymoSelect™ MS Grade Protease at –30°C to –10°C.

2.C. Reconstituted Protease Storage Conditions

Once rChymoSelect™ MS Grade Protease is reconstituted in sterile ultrapure water, store the dispensed aliquots at:

- 4°C for up to 1 week
- –20°C for up to 1 month
- –80°C for up to 6 months

Do not freeze-thaw reconstituted rChymoSelect™ MS Grade Protease more than three times.

3. Before You Begin

3.A. Assay Considerations

To ensure optimal proteolytic activity and high cleavage specificity, perform digestion in HEPES or Tris-HCl buffer (pH 7.5) supplemented with 0.8M guanidine hydrochloride (GuHCl). Maintaining pH 7.5 during digestion is critical for preserving enzymatic activity and minimizing nonspecific cleavages. Mildly denaturing conditions enhance proteolysis by increasing cleavage site access.

Recommended digestion conditions:

- E:S ratio: 1:20 to 1:40
- Buffer: 50mM HEPES or Tris-HCl (pH 7.5)
- Denaturant: 0.8M GuHCl
- Digestion time: 2 hours
- Temperature: 25°C

Note: Dispose of waste according to your institutional guidelines.

3.B. Materials to be Supplied by the User

- sterile ultrapure water (or equivalent grade)
- 500mM HEPES or Tris-HCl buffer (pH 7.5)
- 8M guanidine hydrochloride (GuHCl) or 8M urea
- Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) or dithiothreitol (DTT)
- iodoacetamide (IAM) or chloroacetamide (CAM)
- trifluoroacetic acid (TFA) or formic acid
- microcentrifuge tubes

4. Protocols

4.A. Preparing Protease and Solutions

Preparing Solutions

Use sterile ultrapure water to prepare 8M GuHCl (or 8M urea), 500mM Tris-HCl (pH 7.5), 100mM TCEP, and prepare fresh 300mM IAM. Store IAM solution in the dark until use.

Reconstituting the rChymoSelect™ MS Grade Protease

Only reconstitute the rChymoSelect™ MS Grade Protease when needed in the protocol (Section 4.B or 4.C). Use the following instructions to correctly reconstitute the protease.

1. Reconstitute the protease to 0.25µg/µl using sterile ultrapure water. **Do not** reconstitute with HCl or acetic acid solutions.
 - a. For a 25µg/vial, add 100µl sterile ultrapure water.
 - b. For a 100µg/vial, add 400µl sterile ultrapure water.
2. Dispense into aliquots and store at:
 - a. 4°C for up to 1 week **or**
 - b. -20°C for up to 1 month **or**
 - c. -80°C for up to 6 months
3. To maintain optimal protease activity, do not freeze-thaw reconstituted rChymoSelect™ MS Grade Protease more than three times.

4.B. Protein Digestion for Peptide Mapping Analysis of Biotherapeutic Proteins

Protein Denaturation and Disulfide Bond Reduction (100µg protein)

The following conditions are used to denature and reduce IgG: 5.4M GuHCl, 50mM Tris-HCl (pH 7.5), 3.4µg/µl IgG and 5mM TCEP.

1. Mix the following components:

Reagent	Volume
8M GuHCl	20.0µl
500mM Tris-HCl (pH 7.5)	3.0µl
20µg/µl protein of interest	5.0µl
100mM TCEP	1.5µl
Total	29.5µl

2. Incubate at 37°C for 30 minutes.

Alkylation

1. Add 1.5µl of freshly prepared 300mM IAM to the reduced protein, for a final concentration of 15mM, mix and incubate protected from light at room temperature for 30 minutes. The final concentration of the protein after reduction and alkylation will be 3.22µg/µl.
2. After alkylation is complete, proceed with the next step, Digestion.

Digestion (100µg protein)

The following conditions are used to digest a pre-reduced and alkylated IgG: 0.8M GuHCl, 58mM Tris-HCl (pH 7.5), 0.5µg/µl IgG and 0.025µg/µl rChymoSelect™ protease.

1. Reconstitute the lyophilized rChymoSelect™ MS Grade Protease with sterile ultrapure water to 0.25µg/µl (Section 4.A). The final rChymoSelect-to-protein ratio in the digestion reaction will be 1:20.
2. Add the following components to the reduced and alkylated protein:

Reagent	Volume
Sterile ultrapure water	129µl
500mM Tris-HCl (pH 7.5)	20µl
rChymoSelect™ MS Grade Protease (0.25µg/µl)	20µl
Total	200µl

3. Incubate for 2 hours at 25°C with constant shaking.
4. After digestion is complete, acidify the digestion reaction with 20µl of 20% TFA or 20% formic acid to a final concentration of 2% to terminate proteolysis.
5. Proceed with analysis.

4.C. Complex Protein Extract Digestion for Proteomics Applications

Note: This protocol uses urea as the denaturant, which is standard practice in bottom-up proteomics workflows. Although urea can introduce carbamylation artifacts, these are generally tolerated in proteomics applications unlike in peptide mapping of biotherapeutics, where such artifacts must be avoided. GuHCl may be substituted; however, the concentrations and volumes in this protocol are optimized for urea-based denaturation.

Denaturation and Disulfide Bond Reduction (100µg protein)

The following conditions are used to reduce a protein extract: 6.5M Urea, 50mM Tris-HCl (pH 7.5), ~5µg/µl protein extract and ~5mM TCEP.

1. Mix the following components:

Reagent	Volume
5µg/µl Protein Extract, Intact, solubilized in 6.5M urea/50mM Tris-HCl (pH 7.5)	20µl
100mM TCEP	1µl
Total	21µl

2. Incubate at 37°C for 30 minutes.

Alkylation

1. Add 1µl of freshly prepared 300mM IAM to the reduced protein, for a final concentration of 14mM, mix and incubate protected from light at room temperature for 30 minutes. The final concentration of the protein after reduction and alkylation will be 4.5µg/µl.
2. After alkylation is complete, proceed with the next step, Digestion.

Digestion (100µg protein)

The following conditions are used to digest a pre-reduced protein extract: 4M urea, 55mM Tris-HCl (pH 7.5), 0.5µg/µl protein extract and 0.025µg/µl rChymoSelect™ protease.

1. Reconstitute the lyophilized rChymoSelect™ MS Grade Protease with sterile ultrapure water to 0.25µg/µl (Section 4.A). The final rChymoSelect-to-protein ratio in the digestion reaction will be 1:20.
2. Add the following components to the reduced and alkylated protein:

Reagent	Volume
Sterile ultrapure water	54µl
8M urea	84µl
500mM Tris-HCl (pH 7.5)	20µl
rChymoSelect™ MS Grade Protease (0.25µg/µl)	20µl
Total	200µl

3. Incubate for 2 hours at 25°C with constant shaking.
4. After digestion is complete, acidify the digestion reaction with 20µl of 20% TFA or 20% formic acid to terminate proteolysis.

5. Data Analysis

In addition to cleaving after tyrosine (Y) and phenylalanine (F), rChymoSelect™ MS Grade Protease shows a strong preference for cleavage after leucine (L), particularly within aliphatic motifs such as LL, VL and AL. Enhanced cleavage is also observed at leucine sites flanked by small residues such as serine (S) and threonine (T). Cleavage at other leucine sites depends on digestion time and E:S ratio. Occasional cleavage may occur after selected methionine (M) residues, although less frequently than at leucine sites. In all cases, cleavage is blocked when the residue following the cleavage site (the P1' position, Schechter–Berger nomenclature) is proline (P); the motifs YP, FP, LP and MP are not cleaved.

For MS/MS data analysis, we recommend defining protease specificity as cleavage after the C-terminals of tyrosine (Y), phenylalanine (F), leucine (L) and methionine (M) residues. Search parameters should allow multiple missed cleavages. Analysis of panitumumab IgG digests prepared under the recommended digestion conditions (E:S = 1:40, 2 hours, 25°C and 0.8M GuHCl) showed that peptides containing more than one missed cleavage are rarely observed at tyrosine, phenylalanine and methionine cleavage sites. Leucine cleavage sites exhibit a higher frequency of missed cleavages, with approximately 16% of the total peptide signal associated with peptides containing two missed cleavages (Figure 3). Allowing two missed cleavages captures greater than 99% of the observed peptide signal, whereas peptides containing three or more missed cleavages account for less than 0.2% of the total signal. The distribution of missed cleavages may vary depending on substrate sequence, digestion conditions and E:S ratio.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. Email: techserv@promega.com

Symptom	Causes and Comments
Increased nonspecific cleavages	Digestion time, protease amount or temperature may be too high. Reduce digestion time, use the recommended buffer and pH conditions, or decrease the protease amount per reaction. The sample is contaminated with a nonspecific protease. Denature and reduce the sample to deactivate native proteases prior to digestion.
Incomplete digestion or excessive missed cleavages	Increase incubation time or protease amount. The sample was not properly denatured or reduced. Denature the sample using high concentrations of GuHCl or urea and dilute the denaturing agent to the recommended concentration prior to digestion. Reduce cysteines when appropriate. The denaturing agent inhibits rChymoSelect™ MS Grade Protease. The extent of inhibition depends on denaturant concentration. Dilute the denaturing agent to the recommended concentration or remove the denaturant prior to digestion. If excessive missed cleavages occur at Leu or Met sites, increase the allowed missed cleavages in MS/MS search parameters to 3–4. Unlike cleavage at Tyr and Phe sites, which is highly efficient, cleavage at Leu and Met sites is dependent on digestion reaction conditions and substrate type. When digestion is suboptimal, restricting missed cleavages in the search parameters to 1–2 may cause a substantial proportion of leucine-containing peptides to go unidentified, given that leucine is one of the most abundant amino acids. Note that reduced cleavage at Leu sites may also reflect intentional method optimization (e.g., when longer peptides are desired). In either case, expanding the search window allows the software to match partially cleaved peptides and recover identifications that would otherwise be missed. This is a database search parameter adjustment, not a substitute for optimizing digestion conditions. rChymoSelect™ MS Grade Protease was reconstituted incorrectly. Do not reconstitute the protease with HCl or acetic acid solutions because these acidic conditions can permanently reduce protease performance. Reconstitute the protease with sterile ultrapure water.

Symptom	Causes and Comments
Reduced activity in high denaturant concentrations	Increase incubation time or protease amount.
Excessive short peptides	Leucine is an abundant amino acid, and rChymoSelect™ MS Grade Protease cleaves at leucine sites. At lower protease amounts or shorter digestion times, preferred leucine cleavage sites are targeted. At higher protease amounts or extended digestion times, additional leucine cleavage sites may be cleaved, resulting in increased formation of short peptides (Table 1). Reduce digestion time or decrease the protease amount per reaction.
Nonreproducible LC chromatograms	Reproducibility depends on digestion reaction conditions. Variations in incubation time, temperature or protease amount may alter the extent of digestion, primarily because of differential cleavage at leucine sites. Maintain consistent digestion conditions to improve reproducibility. Reconstituted rChymoSelect™ MS Grade Protease lost activity. Refer to Protease Reconstitution and Storage Conditions Section.

7. Appendix

7.A. Additional Representative Data

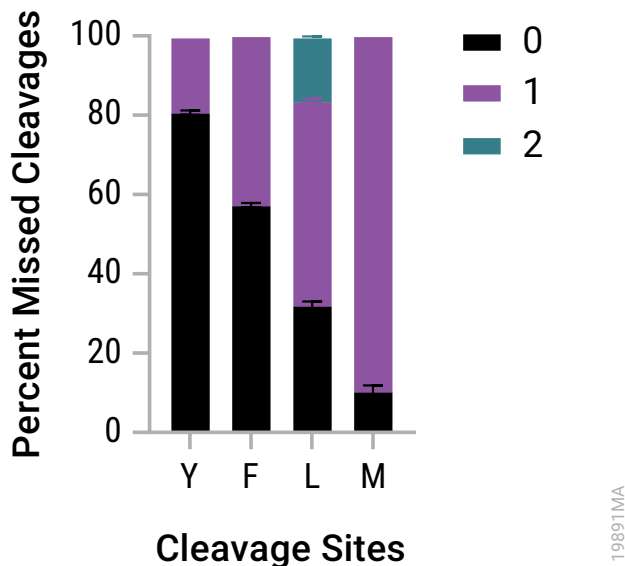


Figure 3. Missed cleavages in rChymoSelect™ MS Grade Protease digests of an IgG. The percentage of total extracted ion chromatogram (XIC) intensity associated with peptides containing 0, 1, 2 or ≥ 3 missed cleavages was determined for tyrosine (Y), phenylalanine (F), leucine (L) and methionine (M) cleavage sites in rChymoSelect™ MS Grade Protease digests ($n = 3$; IgG2 (Panitumumab) digest, E:S = 1:40, 2 hours, 25°C, 0.8M GuHCl). Leucine and methionine cleavage sites show a higher frequency of missed cleavages than tyrosine and phenylalanine cleavage sites. Peptides containing ≥ 3 missed cleavages accounted for less than 0.2% of the total XIC intensity and are not visible at the scale shown. Error bars represent standard deviation.

Supporting Data for Proteomics Applications

Bottom-up proteomics uses proteolytic enzymes to generate peptides suitable for identification by mass spectrometry. Although rChymoSelect™ MS Grade Protease was developed primarily for peptide mapping of biotherapeutic proteins, its unique cleavage specificity and resistance to autoproteolysis also make it suitable for proteomics workflows. The following data demonstrate the performance characteristics of rChymoSelect™ MS Grade Protease in comparison to traditional chymotrypsin and trypsin.

In the following experiments, MS-Compatible Human Protein Extract, Intact (Promega Cat.# V6941), was digested with rChymoSelect™ MS Grade Protease at E:S ratio of 1:40 in the presence of 4M urea. For comparison, trypsin (Cat.# V5280) and traditional chymotrypsin (Cat.# V1061) were used to digest the same protein extract according to the manufacturers' recommended protocols. All digestions were performed in triplicate and analyzed by LC-MS/MS. Peptide and protein identifications were generated using Byonic software (Protein Metrics).

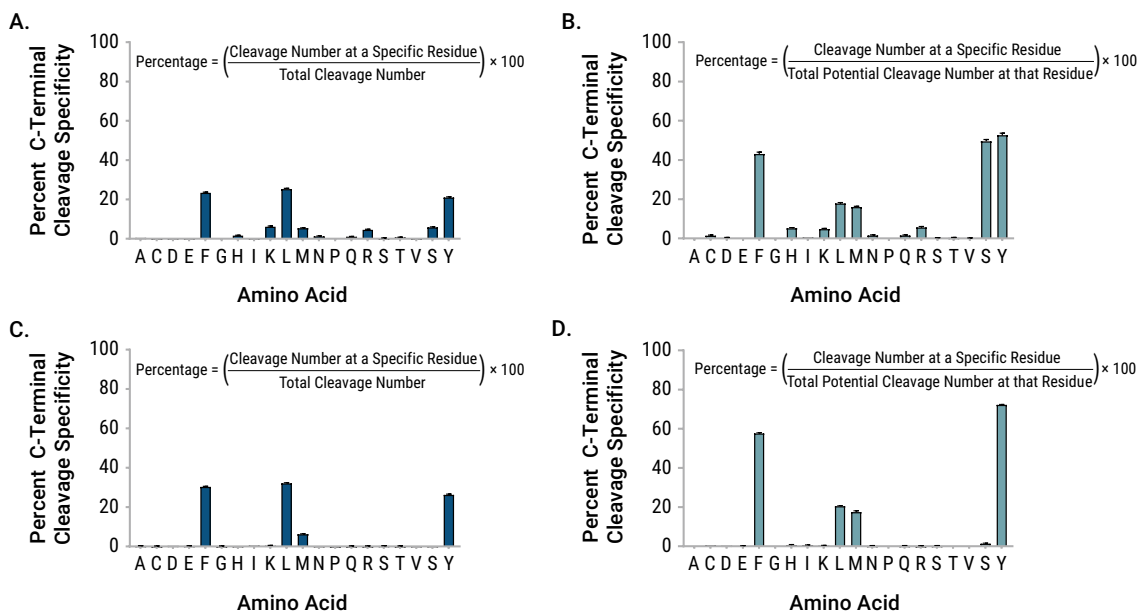


Figure 4. Cleavage specificity and efficiency in a complex mixture digest. Cleavage specificity and cleavage efficiency were determined for digests generated with traditional chymotrypsin (**Panels A and B**) or rChymoSelect™ MS Grade Protease (**Panels C and D**). **Panels A and C** show cleavage specificity, defined as the percentage of total observed cleavages occurring after each amino acid residue. **Panels B and D** show cleavage efficiency, defined as the percentage of potential cleavage sites that were cleaved for each amino acid residue. More than 95% of all cleavages generated by rChymoSelect™ MS Grade Protease occurred after tyrosine (Y), phenylalanine (F), leucine (L) and methionine (M), with minimal cleavage at other residues. In contrast, traditional chymotrypsin generated substantial off-target cleavages after histidine (H), lysine (K), arginine (R), asparagine (N) and glutamine (Q), resulting in a broader and less predictable cleavage profile.

7.A. Additional Representative Data (continued)

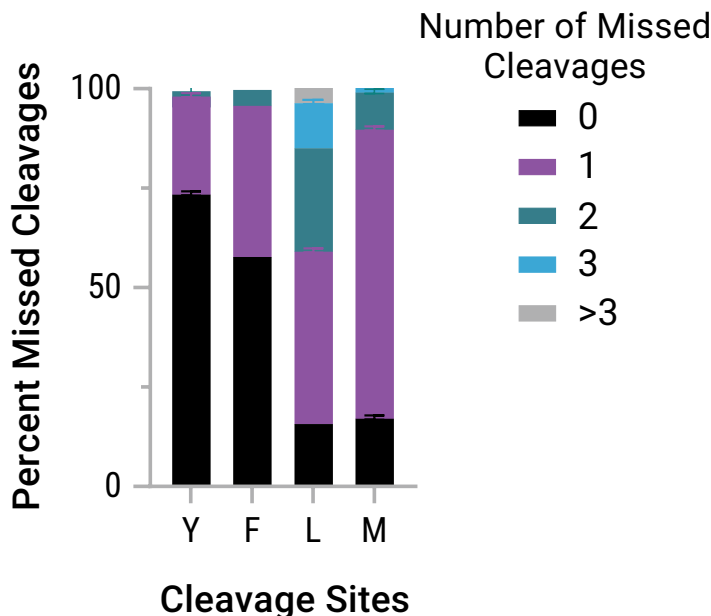


Figure 5. Missed cleavages in rChymoSelect™ MS Grade Protease digests of a complex sample. The frequency of missed cleavages was determined for peptides generated by cleavage after tyrosine (Y), phenylalanine (F), leucine (L) and methionine (M) in human proteome digests prepared with rChymoSelect™ MS Grade Protease. Tyrosine and phenylalanine cleavage sites exhibited high cleavage efficiency, with most identified peptides containing zero or one missed cleavage. In contrast, leucine cleavage sites showed a broader distribution of missed cleavages, including peptides containing up to three missed cleavages. Methionine cleavage sites were predominantly associated with one or two missed cleavages. These results support the use of Y/F/L/M cleavage specificity and allowance of up to three missed cleavages during MS/MS database searching to maximize peptide identification of proteomic experiments.

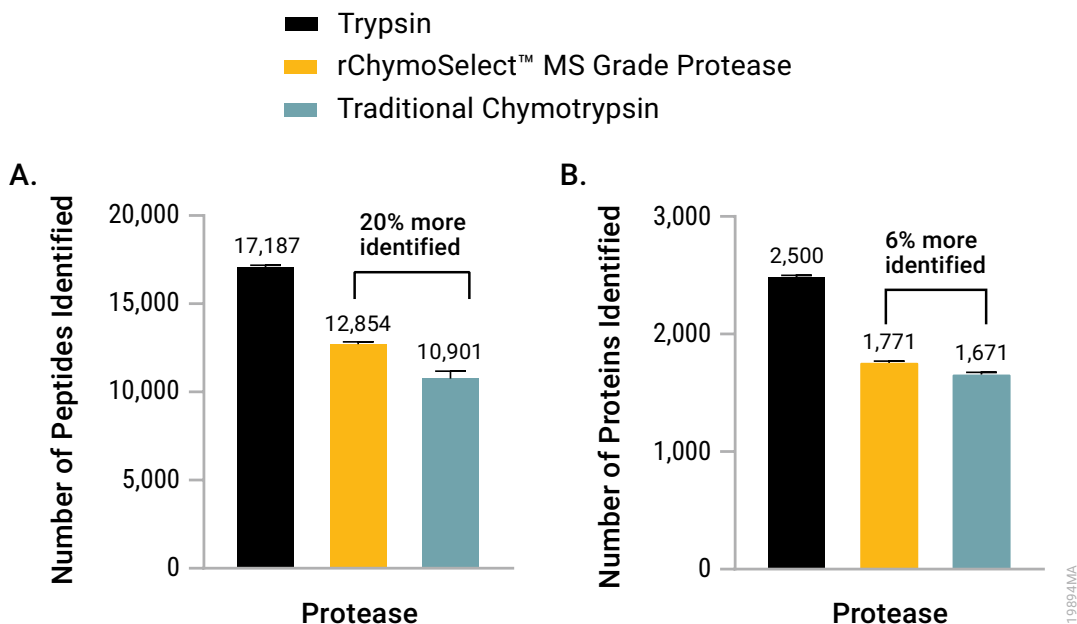


Figure 6. Proteome digestion performance of rChymoSelect™ MS Grade Protease compared with traditional chymotrypsin.

Human proteome digests generated with trypsin, traditional chymotrypsin and rChymoSelect™ MS Grade Protease were analyzed by LC-MS/MS. The number of unique peptide identifications (**Panel A**) and protein identifications (**Panel B**) is shown for each protease. Compared to traditional chymotrypsin, rChymoSelect™ MS Grade Protease produced more unique peptide sequences and more protein identifications. Peptide and protein identifications were generated using Byonic software. Bars represent the mean of three replicates; error bars represent standard deviation.

7.A. Additional Representative Data (continued)

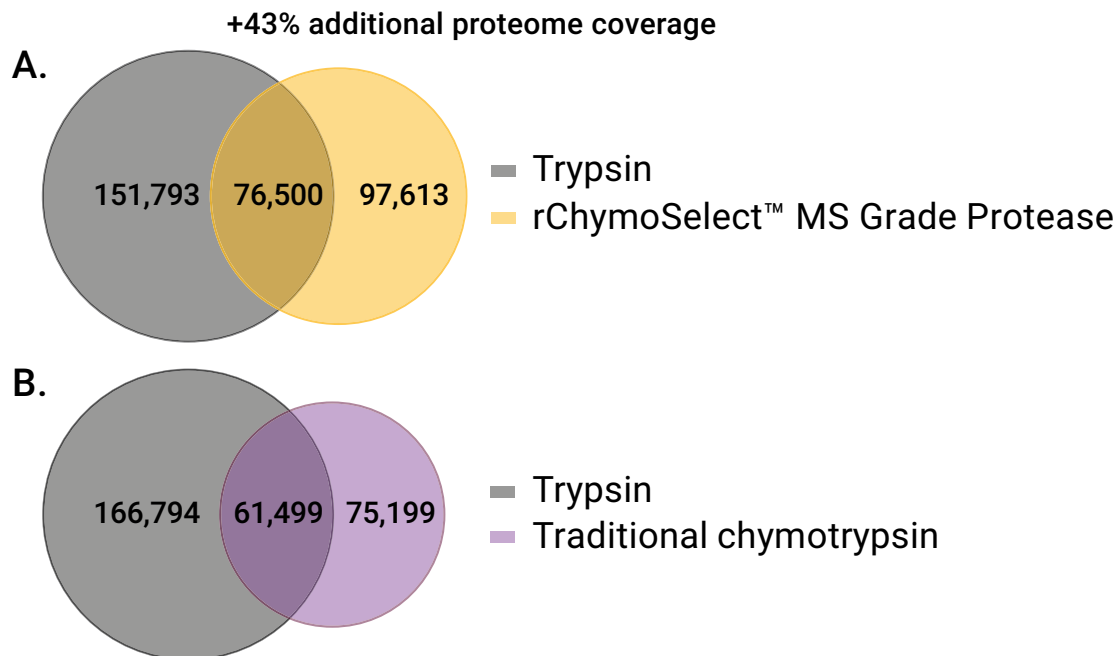


Figure 7. Expanded proteome coverage obtained by combining trypsin and rChymoSelect™ MS Grade Protease digests. Venn diagrams show the overlap in proteome amino acid sequence coverage between trypsin and **(Panel A)** rChymoSelect™ MS Grade Protease or **(Panel B)** traditional chymotrypsin. Numbers indicate unique amino acid residues identified in each dataset. Regions unique to rChymoSelect™ MS Grade Protease or traditional chymotrypsin represent sequence coverage obtained beyond that provided by trypsin alone. Relative to trypsin alone, rChymoSelect™ MS Grade Protease expanded proteome amino acid sequence coverage by approximately 43%, whereas traditional chymotrypsin expanded coverage by approximately 33%. These results demonstrate that rChymoSelect™ MS Grade Protease provides greater complementary proteome sequence coverage than traditional chymotrypsin.

7.B. Experimental Design Considerations

Use the recommended digestion conditions as the starting point for method development. The default workflow was established using panitumumab IgG and 0.8M GuHCl at pH 7.5. Higher GuHCl concentrations may require longer digestion times or increased protease amount to maintain digestion efficiency. rChymoSelect™ MS Grade Protease retains activity in urea, although digestion efficiency may decrease modestly at 6M urea. Missed-cleavage behavior depends on substrate sequence and digestion conditions; leucine sites show more missed cleavages than tyrosine, phenylalanine or methionine sites. For MS/MS database searching, use Y/F/L/M specificity and allow multiple missed cleavages, with greater allowance for leucine-rich peptides.

7.C. Compatibility with common sample additives

Detergents and organic solvents are common components in sample preparation buffers, but they can inhibit protease activity at sufficient concentration. Table 2 lists the highest concentration of five commonly used additives at which rChymoSelect™ MS Grade Protease retained at least 80% or more of the specific activity compared to no additive present. rChymoSelect™ MS Grade Protease tolerates nonionic and mildly anionic detergents at typical working concentrations, along with up to 10% acetonitrile or ethanol, while SDS is inhibitory at much lower concentrations and should be removed or diluted before digestion.

Table 2. Sample Preparation Buffer Additives Compatible with ≥80% rChymoSelect™ MS Grade Protease Activity.

Additive	Maximum Tolerated Concentration for ≥80% Proteolytic Activity
SDS	0.0028% (w/v)
Sarkosyl	0.6% (w/v)
Triton-X	1.9% (v/v)
Acetonitrile	10% (v/v)
Ethanol	10% (v/v)

7.D. Additional Assay Formats

Digestion Following Desalting

Proteins may be denatured, reduced and alkylated as described in Section 4.B, followed by desalting prior to digestion. Removal of GuHCl by desalting may increase rChymoSelect™ MS Grade Protease activity because the denaturant no longer suppresses proteolytic activity. However, removal of the denaturant may also promote partial refolding of the target protein, reducing accessibility of certain cleavage sites. As a starting point for method optimization, digest desalted proteins at an E:S ratio of 1:20 for 1–2 hours at 25°C. Digestion conditions should be optimized for individual proteins.

Digestion Under Mildly Acidic Conditions

Mildly acidic digestion conditions may be advantageous for characterization of non-enzymatic post-translational modifications because they minimize artificial deamidation and disulfide bond scrambling that can occur under neutral to mildly basic digestion conditions. rChymoSelect™ MS Grade Protease retains proteolytic activity under mildly acidic conditions. For peptide mapping workflows intended to characterize non-enzymatic modifications, reduced and alkylated proteins may be digested in MES (pH 6.5) or histidine (pH 6.0) buffers supplemented with 0.8M GuHCl. Based on experiments with panitumumab IgG, an E:S ratio of 1:10 and a digestion time of 2 hours at 25°C provide a suitable starting point for method optimization. Digestion conditions should be optimized for individual proteins.

7.D. Additional Assay Formats (continued)

Digestion in High-Denaturant Conditions

rChymoSelect™ MS Grade Protease remains active in the presence of GuHCl and urea. Digestion efficiency decreases as GuHCl concentration increases above the recommended 0.8M concentration. If higher GuHCl concentrations are required to maintain protein denaturation, digestion efficiency may be restored by increasing digestion time and/or protease amount. For urea, a concentration of 4M is recommended; up to 6M urea can be used with minor loss of activity.

7.E. Bibliography

1. Adoni, K.R. *et al.* (2026) Enhanced proteomics analysis with a novel recombinant chymotrypsin analogue engineered for high cleavage specificity. *J. Proteome Res.* **25**, 2511–19.
2. Turner, N.P. *et al.* (2026) Rapid histone post-translational modification analysis using alternative proteases and tandem mass tags. *Anal. Chem.* **98**, 18694–712.

^(a)Patents Pending.

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