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Application Note

*Rapi*Gest SF Surfactant: An Enabling Tool for In-Solution Enzymatic Protein Digestions

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Abstract

In this application note, we provide an overview of the physical and chemical characteristics of Waters' patented *Rapi*Gest SF and illustrate selected application areas. First introduced in 2002 as an enzyme-friendly surfactant to assist in-solution protein digestion, *Rapi*Gest SF is an innovative product created to improve protein solubility during sample preparation.

Introduction

In this application note, we provide an overview of the physical and chemical characteristics of Waters' patented *Rapi*Gest SF and illustrate selected application areas. First introduced in 2002 as an enzyme-friendly surfactant to assist in-solution protein digestion, *Rapi*Gest SF is an innovative product created to improve protein solubility during sample preparation.

The mechanism by which RapiGest SF improves the speed and completeness of digestion is illustrated in Figure 1. Mild protein denaturation opens protein structure and exposes the proteolytic sites to enzymatic cleavage. Enzymes are more resistant to denaturation than common proteins and remain active in *Rapi*Gest SF solutions. More complete denaturation of globular proteins can be accomplished by heating in a *Rapi*Gest SF solution at elevated temperatures prior to the addition of enzymes and incubating the sample with the enzyme at 37 °C.[1,2]

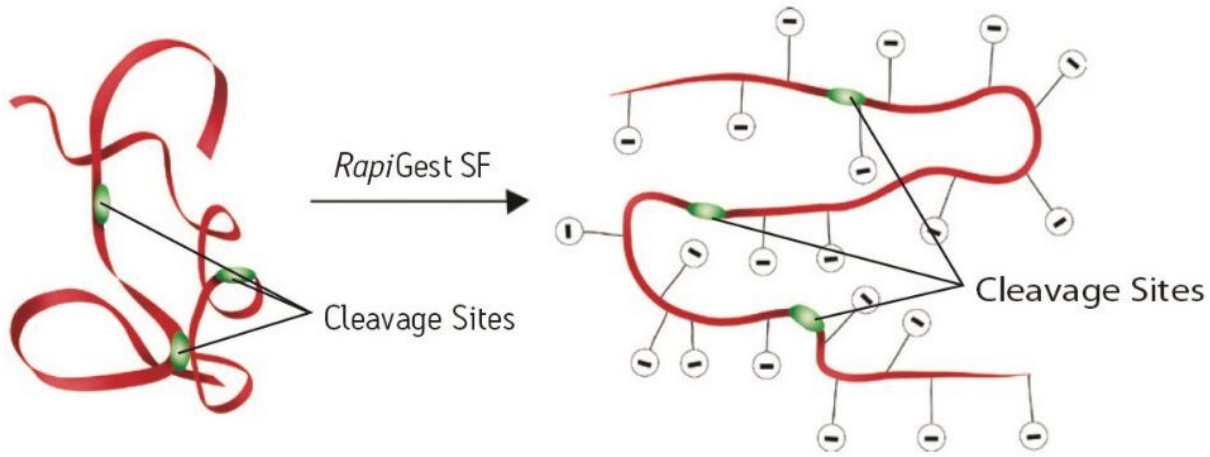


Figure 1. The protein substrate unfolds in RapiGest SF solution and becomes more amenable to proteolytic cleavage.

More than 200 peer-reviewed journals have cited the use of *Rapi*Gest SF for general sample solubilization purposes, mostly for proteomic applications. Recently, many pharmaceutical laboratories have adopted *Rapi* Gest SF for biotherapeutic protein characterization. Because of improved digestion and easy surfactant removal prior to LC and MS analysis, *Rapi*Gest SF has become widely accepted for many applications, including high sequence coverage LC-UV/MS peptide mapping of therapeutic proteins.

Experimental

LC conditions

LC System:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH 300 C_{18}, Peptide Separation Technology Column, 2.1 x 100mm
Column temp.:	40 °C

Sample injected:	2 µL (10 pmol)
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in acetonitrile
Flow rate:	200 µL/min
Gradient:	0 to 2 min: 2% B 2 to 92 min: 2 to 35% B 92 to 102 min: 35 to 50% B 102.1 to 105 min: 90% B 105.1 to 110 min: 2% B

MS conditions

MS System:	SYNAPT MS (V mode)
Capillary voltage:	3.2 kV
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas:	700 L/hr
MS scan rate:	1 sec/scan
Lock Mass channel:	100 fmol/µL Glu-Fib peptide (*m/z* 785.8426, z = 2), flow rate 20 µL/min

Results and Discussion

*Rapi*Gest SF is an acid labile surfactant that undergoes hydrolysis in acidic conditions.[1] This unique feature can be utilized to remove the surfactant from solutions when desired. The structure of *Rapi*Gest SF and its byproducts from the acid hydrolysis are shown in Figure 2. The acid labile property facilitates a nearly complete surfactant degradation of within about 45 minutes at pH 2.[1]

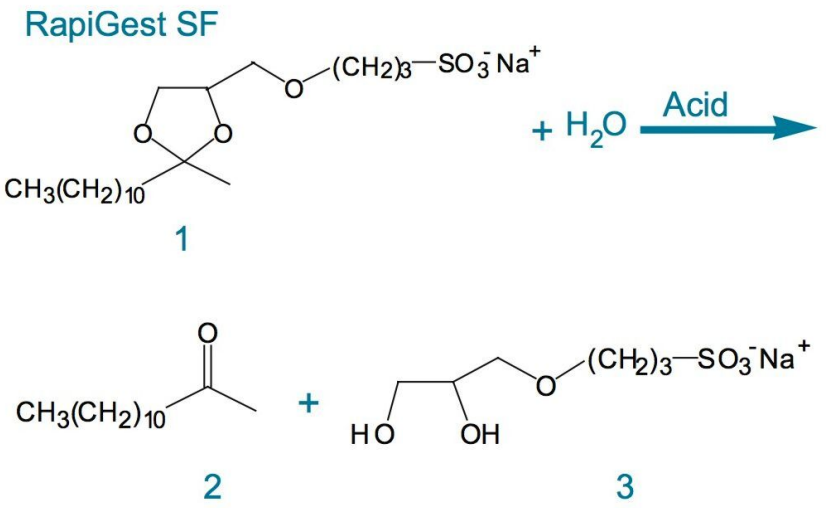


Figure 2. RapiGest SF (1) degrades in acidic solution to (2) and (3). The t1/2 is 7.6 minutes at pH 2.

The surfactant decomposes into two products, dodeca-2-one and sodium-3-(2,3-dihydroxypropoxy) propanesulfonate. The first compound is water immiscible, and can be removed by centrifugation. The second degradant is highly soluble in aqueous solutions and practically unretained in reversed-phase LC mode. The aqueous fraction of enzymatic digest can be directly analyzed by HPLC, LC-MS, or by MALDI-TOF MS.

Removal after digestion

No additional detergent removal steps are required (e.g., dialysis) prior to sample analysis. The enzymatic digestions are typically acidified with acids such as formic acid, trifluoroacetic acid (TFA), or hydrochloric acid (HCl) to degrade *Rapi*Gest SF prior to further analysis. The recommended pH for degradation is ≤ 2.

Compatibility with tryptic digestion

Trypsin is the most common proteolytic enzyme used for peptide mapping and proteomic applications. We have investigated trypsin activity in the presence of *Rapi*Gest SF and compared it to most common denaturants cited in literature. The assay was based on trypsin induced hydrolysis of N-α-benzoyl-L-arginine ethyl ester (BAEE) in 50 mM ammonium bicarbonate (pH 7.9) at room temperature. Changes in trypsin activity were calculated by measuring the rate of BAEE hydrolysis at UV 253 nm. Trypsin activity in the selected denaturant solution was compared against the control sample (no denaturant). The results are shown in Table 1.

Trypsin solution[A]	Trypsin activity[B] (%)	Trypsin solution[A]	Trypsin activity[B] (%)
No additive	100	50% Methanol	31
0.1% RapiGest	100	50% Acetonitrile	92
0.5% RapiGest	87	1 M Urea	97
0.1% SDS	20	2 M Urea	83
0.5 SDS	1	0.5 M Guanidine HCl	21
0.1 RapiGest/0.1% SDS	58	1 M Guanidine HCl	8

Table 1. Trypsin activity measured in the presence of selected denaturants. A. 0.5 µg of trypsin was added to 1 mL of 50 mM ammonium bicarbonate, pH 7.9, containing 0.2 mM of BAEE. B. Measured as delta BAEE absorbance at 253 nm (slope within 5 minutes).

The data in Table 1 suggest that at low concentrations (0.1%), *Rapi*Gest SF does not inhibit trypsin activity. This contrasts with structurallysimilar surfactant SDS, which appears to be a strong denaturant and inactivates the trypsin. Urea, acetonitrile, or guanidine-HCl were also proposed as denaturants for tryptic digestions. However, acetonitrile is a strong eluent and interferes with reversed-phase LC analysis of digested sample. Urea is known to cause covalent modification of proteins, and Guanidine-HCl inactivates enzyme, similarly to SDS.

The implication from this experiment is that enzyme proteolysis activity can be affected by the denaturant used to solubilize the protein samples. Using *Rapi*Gest SF at low to high concentrations does not alter the enzyme activity; therefore, optimum proteolysis digestion is achieved without using an excess of enzyme.

Fast proteolytic digestions

Proteins that are resistant to proteolysis can be digested within minutes using *Rapi*Gest SF. A complete

protein digestion for a globular protein, horse myoglobin, was achieved within 5 minutes. The comparison of results for surfactant-aided and control digestion is shown in Figure 3. Due to its globular nature, myoglobin is known to be difficult to digest without the use of any denaturant. In the control reaction, only a small fraction of protein is digested after 9 hours of incubation with trypsin. The overall digestion efficiency significantly improved when using *Rapi*Gest SF.

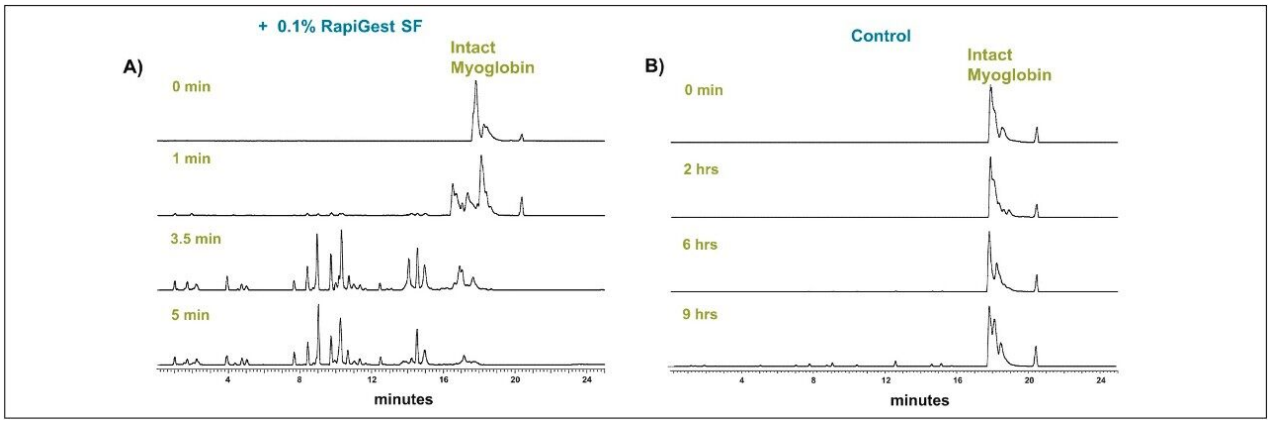


Figure 3. LC-MS total ion chromatograms of tryptic digest of horse myoglobin, (A) solubilized with 0.1% Rapi Gest SF, and (B) control digestion (no denaturant). Myoglobin digestion in 0.1% RapiGest SF solution provided complete tryptic digestion within 5 minutes, while control digestion remains incomplete even after 9 hours.

Improved sequence coverage in peptide mapping of therapeutic proteins

*Rapi*Gest SF has been widely used in proteomics sample preparation as an effective denaturant for protein solubilization. Recently, more biopharmaceutical labs have adopted *Rapi*Gest SF in their peptide mapping protocols. Several publications document the benefits of using *Rapi*Gest SF for therapeutic protein digestion.[4,5] The reported *Rapi*Gest SF concentration used ranges from 0.05 to 1% depending on the protein hydrophobicity and concentration.

We have found that a 0.05 to 0.1% concentration of *Rapi*Gest SF is sufficient to denature various sizes of proteins; higher concentration of *Rapi*Gest SF may be suited for a whole cell protein extraction type of experiment.

Peptide mapping of monoclonal antibodies (mAbs) is challenging due to the difficulty of digesting these large and hydrophobic proteins. The goal of peptide mapping analysis is to confirm the protein sequence and identify all present post-translational modifications (PTMs). Figure 4 shows an example of *Rapi*Gest SF-assisted digestion of humanized mAb. The parameters of sample preparation and analysis by UPLC and

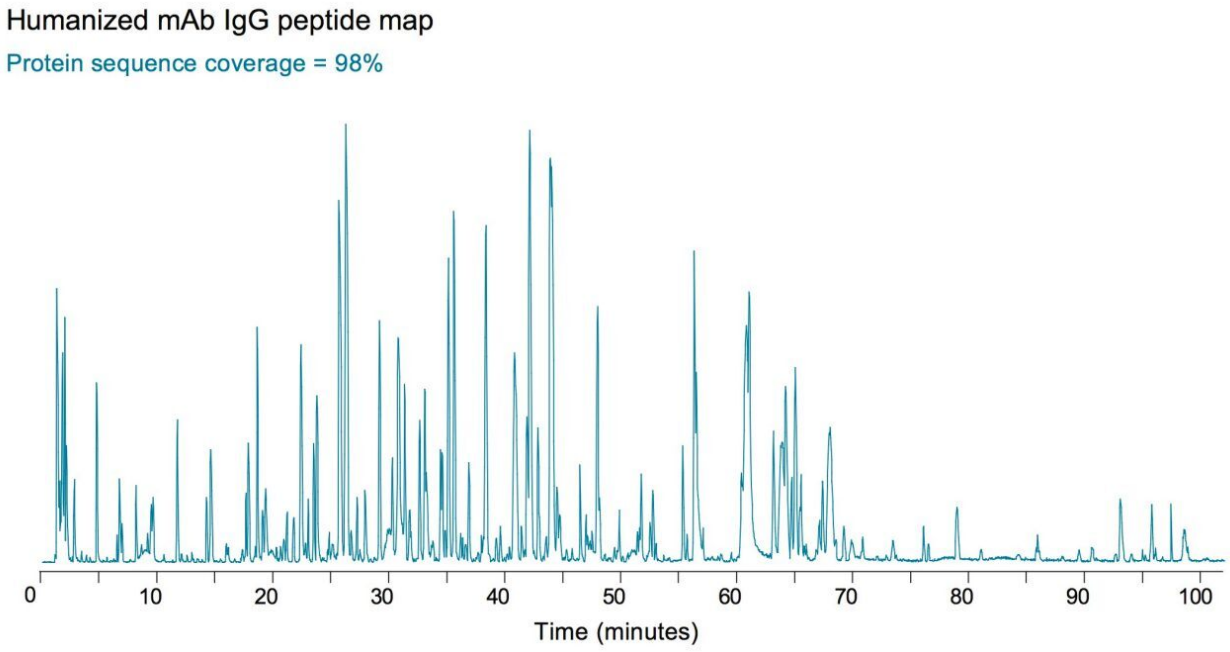
quadrupole time-of-flight MS are listed as guidelines.

The overall sequence coverage in the experiment shown in Figure 4 was 98%. Data analysis was performed with BiopharmaLynx Software, v.1.2. The high sequence coverage (98%) indicates a complete digestion of mAb. No intact protein or large miscleaved peptides were detected in LC-MS analysis. The remaining 2% of unaccounted sequence belong to a few two amino-acid-long peptides or to a single amino acid (R or K) that are unretainable on the reversed-phase column.

Sample preparation

Humanized mAb sample (10 µL, 21 mg/mL) was solubilized in 50 µL 50 mM ammonium bicarbonate containing 0.1% (w/v) *Rapi*Gest SF. 2 µL of 0.1 M dithiothreitol (DTT) was added to the sample, and the sample was heated at 50 °C for 30 minutes. 4 µL of 0.1 M iodoacetamide was added to the sample, after it was cooled to room temperature, and the sample was placed in the dark for 40 minutes.

8 µg of trypsin was added to the sample (trypsin conc. = 1 µg/µL) and the sample was incubated at 37 °C overnight. The digested sample was mixed with 1% formic acid in 10% acetonitrile (1:1, v:v). The sample was diluted to 5 pmol/µL with Milli-Q water (Millipore) prior to LC-MS analysis.



Use with additional proteolytic enzymes

We tested *Rapi*Gest SF compatibility with multiple proteolytic enzymes, for example, Asp-N, Lys-C, and Glu-

C. Efficient digest results were obtained using *Rapi*Gest SF to denature the protein prior to proteolysis (Figure 5).

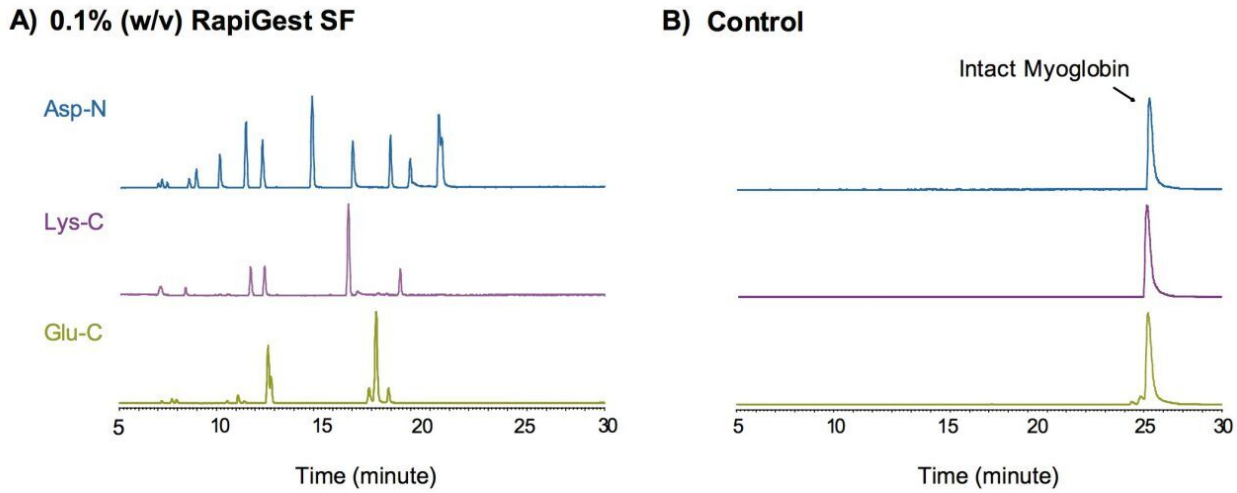


Figure 5. Horse myoglobin (50 pmol/µL) digestion with Asp-N, Lys-C, and Glu-C, with or without 0.1% (w/v) RapiGest SF. A. LC-MS analysis after 1 hour incubation at 37 °C with 0.1% RapiGest SF; no intact protein was left undigested. B. The control experiment (no surfactant) showed that majority of the myoglobin remains undigested.

Use for protein deglycosylation

*Rapi*Gest SF was also tested with other enzymes such as PNGase F, which is used to cleave N-linked glycans from glycoproteins.[2] Figure 6 illustrates the deglycosylation of chicken ovalbumin. Complete deglycosylation was observed after 2 hours in *Rapi*Gest SF-mediated digestion with PNGase F.

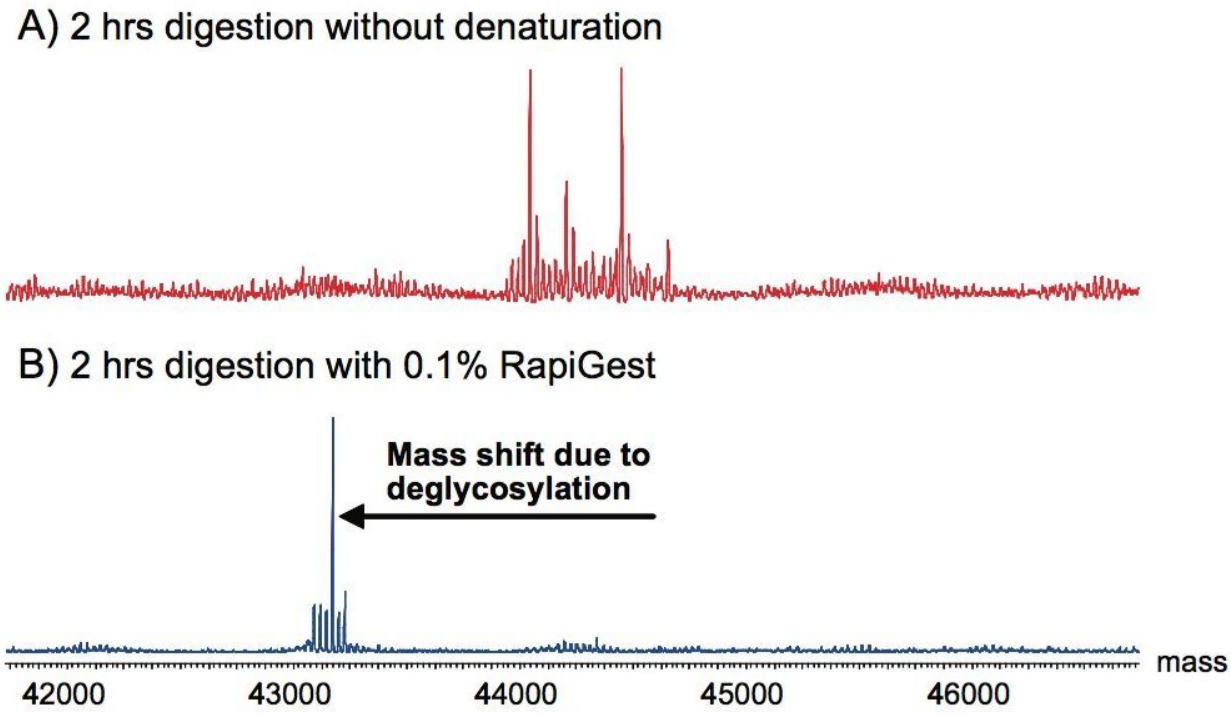


Figure 6. Chicken ovalbumin was deglycosylated with PNGase F without denaturation (A) and with 0.1% of RapiGest SF (B). The main signal in the deconvoluted LC-MS spectrum (B) represents ovalbumin without glycans (MW is consistent with amino acid composition). The heterogeneous MS signals in deconvoluted spectrum (A) indicates the presence of several glycoforms. The majority of glycans were not released from protein even after 2 hours of digestion. For details, see reference 3.

Conclusion

- *Rapi*Gest SF improves the speed and completeness of protein enzymatic digestions, enabling high-sequence-coverage peptide mapping of therapeutic proteins.
- *Rapi*Gest SF is a proven denaturant suitable for proteomic, glycomic, and biotherapeutic application areas.
- Minimal or no post-digestion sample preparation is required. Simple acidification of the sample is sufficient to remove *Rapi*Gest SF from solutions. In many cases, a simple dilution is acceptable prior to LC-MS analysis.

- *Rapi*Gest SF simplifies preparation protocols and improves throughput of analyses: its use improves laboratory productivity and overall data quality.

References

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2. Yu YQ, Gilar M, Lee PJ, Bouvier ES, Gebler JC, A complete peptide mapping of membrane proteins: a novel surfactant aiding the enzymatic digestion of bacteriorhodopsin. Rapid Commun. Mass Spectrom. 2004; 18: 711-715.
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720003102, June 2009

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