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1.0 Purpose

This procedure describes the conditions for enzymatic digestion of macromolecule samples in preparation for peptide mapping, mass spectrometry, SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) and other bioanalytical techniques.

2.0 Scope

This procedure applies to Process Analytics/Quality Control (PA/QC) personnel.

3.0 Authority and Responsibility

3.1 The Manager, Technical Operations, Process Analytics/Quality Control (PA/QC) has the authority to define this procedure.

3.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA/QC personnel are responsible for the performance of this procedure.

3.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.

3.5 BQA is responsible for quality oversight of this procedure.

4.0 Materials and Equipment

4.1 Sequencing Grade Modified Trypsin, BDP PN 30330 or equivalent.

4.2 Sequencing Grade Chymotrypsin, BDP PN 30343 or equivalent.

4.3 Ammonium bicarbonate, BDP PN 30331, or equivalent.

4.4 1M Tris-HCl Buffer, pH 8.0, BDP PN 10097, or equivalent.

4.5 Guanidine hydrochloride (HCl), BDP PN 10064 or equivalent.

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4.6 Urea, BDP PN 10063 or equivalent.

4.7 1,4-Dithiothreitol (DTT), BDP PN 30332 or equivalent.

4.8 Hydrochloric acid (HCl), 1.0N, BDP PN 10020 or equivalent.

4.9 Acetonitrile, HPLC Grade, BDP PN 30075 or equivalent.

4.10 Iodoacetic acid, BDP PN 30366 or equivalent.

4.11 0.5 mL microcentrifuge tubes, BDP PN 21369 or equivalent.

4.12 1.5 mL microcentrifuge tubes, BDP PN 20595 or equivalent.

4.13 Mini-Spin microcentrifuge, Eppendorf PN 022620100 or equivalent.

4.14 High quality water (which includes deionized, reverse-osmosis, Milli-Q, WFI or other purified water).

5.0 Introduction

While there are many digest enzymes available with various specificities, this procedure will focus on two (2) commonly used enzymes, modified trypsin, and chymotrypsin.

Modified Trypsin: Trypsin is a serine protease that specifically hydrolyzes peptide bonds at the carboxylic sides of lysine and arginine residues. The trypsin listed in Section 4.1 has been modified by reductive alkylation, rendering it extremely resistant to autolytic digestion. There are some restrictions to the specificity of trypsin. When proline is at the carboxylic side of lysine or arginine, the bond is almost completely resistant to cleavage by trypsin. Cleavage may also be considerably reduced when acidic residues are present on either side of a potentially susceptible bond. Modified trypsin is maximally active in the range of pH 7-9 and is reversibly inactivated at pH 4. It is resistant to mild denaturation conditions, including 0.1% SDS, 1M urea and 10% acetonitrile; 48% activity is retained in the presence of 2M guanidine hydrochloride.

Chymotrypsin: Chymotrypsin is a serine protease that specifically hydrolyzes peptide bonds at the carboxylic sides of tyrosine, phenylalanine, and tryptophan residues. In addition, chymotrypsin cleaves at the carboxylic sides of leucine, methionine, alanine, aspartic acid, and glutamic acid at a lower rate. Chymotrypsin is maximally active in the range of pH 7-9. It is resistant to mild denaturation conditions, including 1M urea, 1M guanidine hydrochloride and 5% acetonitrile, with activity reduced by 20% maximally (with a five (5) hour incubation time). The remaining activity after one (1) hour incubation in 0.01% SDS is 50-60%; in the presence of ≥ 0.1% SDS, chymotrypsin is not active.

6.0 Procedure

NOTE: The following procedure applies to both digest enzymes except where noted. It should also be noted that specific sample preparation and digest conditions are dependent upon the test article. Several evaluations of any given protein may be necessary to determine ideal conditions for complete and reproducible digestion results.

6.1 The sample(s) to be digested must be available at an ideal protein concentration of 1 mg/mL (± 0.5 mg/mL) in a formulation that does not inhibit enzymatic digestion (pH 7-9). Many compounds used for product formulations, including sodium chloride, sodium phosphate, Tris, mannitol, citrate, and many other compounds, do not inhibit enzymatic activity, provided that they are present in a fairly low concentration (< 250 mM, arbitrarily). If there is uncertainty as to whether a specific compound may inhibit enzymatic activity, refer to the product insert or contact the manufacturer.

6.1.1 When the sample formulation is not ideal for enzymatic digestion, or if the formulation contains a questionable compound, the sample must first be desalted/buffer exchanged. This can be performed as per SOP 22141 – Desalting and Buffer Exchange of Products Using illustra NAP-5 Columns or SOP 22142 – Desalting, Buffer Exchange and Concentration of Samples Using Centrifugal Filter Devices. Ideal exchange buffers for enzymatic digestion include 50mM Tris-HCl/1mM CaCl2, pH 7.6 (included with modified trypsin), 50mM ammonium bicarbonate (make from stock; pH will be ~8 without adjustment) or 100mM Tris-HCl, pH 8.0 (dilute from stock with high-quality water).

6.1.2 To achieve a target protein concentration of 1 mg/mL, the buffer exchanged sample may have to be concentrated as per SOP 22916 – Operation of the Savant Automatic Environmental SpeedVac.

6.1.3 If the sample(s) to be digested is already available in a formulation that does not inhibit enzymatic digestion, it can either be concentrated to 1 mg/mL (provided that the concentration of other formulation compounds does not exceed the limit as per Section 6.1), diluted to 1 mg/mL using one of the exchange buffers, or dried completely using the SpeedVac and resuspended in one of the exchange buffers.

6.2 Be sure to keep track of the original amount of material used for buffer exchange and/or drying/concentrating, so that the final concentration will be at 1 mg/mL in one of the exchange buffers. The choice of exchange buffer may vary, depending on the product to be digested. A minimum of 100 µg (100 µL at 1 mg/mL) of the sample(s) to be digested should be prepared and transferred to a 0.5 or 1.5 mL microcentrifuge tube.

6.3 Prepare the lyophilized digest enzyme(s) by resuspending with the exchange buffer used for sample preparation (or the sample formulation buffer if no sample preparation was needed) to a concentration of 1 mg/mL (20 µL for modified trypsin; 25 µL for chymotrypsin). Vortex gently until the powder is in solution. Either one (1) or both enzymes can be prepared for protein digestion, depending on the desired application. These preparations are only good for single use applications (enzyme remains active for up to 24 hours when stored at 2-8°C following use). Alternatively, the digest enzymes can be resuspended for longer-term, multiple use storage as follows:

6.3.1 Modified Trypsin: reconstitute with 20 µL of 50mM acetic acid (supplied with enzyme) for storage for up to three (3) months at -20°C. Avoid multiple freeze/thaw cycles or exposure to frequent temperature changes that can impact product stability. Prepare prior to initial use, and then freeze immediately. On reuse, quick-thaw the enzyme and then quickly refreeze the unused portion to maintain maximum activity.

6.3.2 Chymotrypsin: reconstitute with 25 µL of 1mM (0.001N) HCl (dilute from stock with high quality water) for storage for up to one (1) week at 2-8°C.

6.4 If preparing a sample for digestion using both enzymes, separate the sample into two (2) (50 µL minimum) aliquots, one (1) for each digest enzyme.

6.5 Prepare an autolysis control blank for each enzyme used by adding exchange buffer (or sample formulation buffer) to a blank microcentrifuge tube in a volume equivalent to that of the test sample. This is to identify any potential digest enzyme autolysis byproducts that may also be present in the sample(s).

6.6 To each sample and autolysis control blank, add the corresponding digest enzyme in an amount equal to 1:30 (w/w) of the total amount/volume (the working range for the enzyme: substrate ratio is 1:100 to 1:20 for modified trypsin and 1:200 to 1:20 for chymotrypsin; variation in the enzyme concentration may be necessary and is dependent upon the sample). For example, if the total sample amount is 50 µg, add 1.67 µL of the corresponding digest enzyme.

6.7 Transfer the sample tubes to the Thermomixer R and incubate while vortexing at 900-1200 rpm, as per ***SOP 22910 - Operation of the Eppendorf Thermomixer R***. Typically, an overnight (up to 18 hrs.) incubation at 37°C will yield complete digestion of most protein samples. Conditions for modified trypsin digestions include 1-24 hour incubation at 32-37°C. Conditions for chymotrypsin digestions include 2-18 hour incubation at 25-37°C. Reducing the incubation temperature may decrease the digestion rate. To determine the extent of digestion, pull a small aliquot of the sample at various time intervals over the course of incubation (2 hours, 4 hours and/or 8 hours) and subject the portion to analysis by High Pressure Liquid Chromatography (HPLC), mass spec or other bioanalytical technique.

6.8 After complete digestion, remove the sample(s) from the shaker/incubator and place in the microcentrifuge. Spin for 30-60 seconds at 4000-6000 rpm to collect any condensate that has collected on the top and sides of the microcentrifuge tube.

6.9 Terminate the protease activity by adding 0.5M (N) HCl (diluted from stock with high quality water) in an amount equal to 10% of the total volume for a final concentration of approximately 50mM HCl. For example, if the total volume is 50 µL (initial sample volume) + 1.67 µL (digest enzyme) – 10 µL (aliquot remove to determine extent of digestion), then the total volume is 41.67 µL. Add 4.17 µL (10% of total) of 0.5M HCl. Briefly vortex the sample(s) and store at 2-8°C until further analysis.

7.0 Suggestions for Problematic Samples

7.1 Some proteins may be resistant to proteolysis under the conditions outlined in this procedure. There are several sample preparation techniques that can be utilized to expose the cleavage sites of the problematic protein to the digest enzyme, to maximize digestion efficiency.

7.2 One solution is to unfold the protein prior to digestion as follows:

7.2.1 After desalting/buffer exchanging (if necessary; as per section 6.1 – 6.2), dry the sample completely using the SpeedVac.

7.2.2 Resuspend the dried pellet using either 6M guanidine HCl/0.1M Tris-HCl, pH 8.0 (make from stock with 0.1M Tris-HCl) or 6M urea/0.1M Tris-HCl, pH 8.0 (make from stock with 0.1M Tris-HCl) with a volume equal to 1/6 (16.7%) of the starting volume prior to drying. Vortex for thirty (30) seconds.

7.2.3 Incubate at 50-60°C while vortexing at 900-1200 rpm for 50-60 minutes using the Thermomixer R.

7.2.4 Remove the sample from the shaker/incubator and allow 10-15 minutes for the sample to cool to room temperature.

7.2.5 Dilute the sample with 0.1M Tris-HCl, pH 8.0 in an amount equal to 5/6 (83.3%) of the original starting volume, so that the final volume is at 1 mg/mL in 1M guanidine HCl (or urea)/0.1M Tris-HCl, pH 8.0.

7.2.6 Proceed with enzymatic digestion starting at Section 6.3.

7.3 If the sample preparation procedure outlined in Section 7.2 is unsuccessful at complete protein digestion, another solution is to perform in-gel digestion as follows:

7.3.1 Perform SDS-PAGE under reducing conditions as per ***SOP 22101 – SDS-PAGE Gel Electrophoresis Using Tris-Glycine Gels*** followed by Coomassie blue staining. Be sure to load at least four wells with 4-6 µg of the test sample.

7.3.2 After destaining the gel, cut out the bands of interest using a scalpel, cut each piece in half, and combine the gel pieces in a 1.5 mL microcentrifuge tube.

7.3.3 Destain the gel pieces by adding 0.5 mL of 0.1M ammonium bicarbonate/acetonitrile (1:1) and vortexing at 900-1200 rpm while incubating at 37°C ± 2°C using the Thermomixer R. Destaining will need to be repeated several times for 30-60 minutes to remove the residual stain.

7.3.4 Dehydrate the gel pieces by adding 250 µL of acetonitrile, vortexing for 10-15 seconds, and letting sit at ambient temperature for 5-10 minutes. Remove liquid using a pipettor and discard in a hazardous waste container. Be careful not to remove any of the gel pieces while discarding the liquid.

7.3.5 Dry the gel pieces completely using the SpeedVac (drying time should be ~10 minutes at a medium dry rate).

7.3.6 Resuspend the digest enzyme(s) to a concentration of 25 ng/µL, using 50mM ammonium bicarbonate (0.8 mL for modified trypsin, 1.0 mL for chymotrypsin).

7.3.7 Rehydrate the dried gel pieces with the digest enzyme using the minimum volume needed for hydration only. Typically, 30-50 µL is sufficient for rehydration.

7.3.8 Add an additional 50 µL of 50mM ammonium bicarbonate to the rehydrated gel pieces.

7.3.9 Incubate at 37°C ± 2°C for 12-18 hours while vortexing at 900-1200 rpm using the Thermomixer R.

7.3.10 After incubation, centrifuge at 4000-6000 rpm for 30-60 seconds.

7.3.11 Remove the supernatant and transfer to a new 0.5 mL microcentrifuge tube.

7.3.12 Extract the gel pieces by adding 50 µL of 50mM ammonium bicarbonate, vortexing for 30-60 seconds and letting sit at ambient temperature for 5-10 minutes. Remove the liquid and add it to the microcentrifuge tube from Section 7.3.11.

7.3.13 Extract the gel pieces by adding 50 µL of 50mM HCl, vortexing for 30-60 seconds and letting sit at ambient temperature for 5 - 10 minutes. Remove the liquid and add it to the microcentrifuge tube from section 7.3.11.

7.3.14 Extract the gel pieces by adding 50 µL of 50mM HCl/acetonitrile (1:1), vortexing for 30 - 60 seconds and letting sit at ambient temperature for 5-10 minutes. Remove the liquid and add it to the microcentrifuge tube from section 7.3.11.

7.3.15 Extract the gel pieces by adding 50 µL of acetonitrile, vortexing for 30-60 seconds and letting sit at ambient temperature for 5-10 minutes. Remove the liquid and add it to the microcentrifuge tube from section 7.3.11.

7.3.16 Discard the remaining gel pieces in a biomedical waste container.

7.3.17 Dry the collected supernatant pool completely using the SpeedVac.

7.3.18 Resuspend the dried pellet with 50 µL of 50mM ammonium bicarbonate and vortex until the material is in solution. Store the sample(s) at 2-8°C until further analysis.

7.4 If neither of the procedures outlined previously results in complete protein digestion, another option is to perform a reduction/alkylation step prior to enzymatic digestion. This will allow cleavage of potential disulfide bonds, followed by blocking of reduced cysteines to prevent any side-chain reactions from occurring. The procedure is as follows:

7.4.1 Prepare the test sample(s) as per Section 7.2. For Section 7.2.2, add DTT to the guanidine HCl (or urea) formulation so that the final concentration is 10mM DTT (6M guanidine HCl (or urea)/10mM DTT/0.1M Tris, pH 8.0). Proceed as per Sections 7.2.3 and 7.2.4.

7.4.2 Prepare a stock solution of iodoacetamide (0.5M iodoacetamide in 6M guanidine HCl (or urea)/1M Tris, pH 8.0).

7.4.3 Add iodoacetamide stock solution to the sample(s) in a volume equal to 10% of the total volume for a final concentration of 50mM iodoacetic acid.

7.4.4 Incubate the sample(s) at room temperature, in the dark, for 45-50 minutes.

7.4.5 Optionally, the reduced and alkylated sample(s) can be buffer exchanged as per Section 6.1.1 to remove excess reagents.

7.4.6 Proceed with Section 7.2.5.

7.5 If the samples were prepared for in-gel digestion (Section 7.3), perform the reduction/alkylation step as follows:

7.5.1 After Section 7.3.5, rehydrate the dried gel pieces with 30-50 µL of 10mM DTT/50mM ammonium bicarbonate.

7.5.2 Incubate at 50-60°C for 50-60 minutes while vortexing at 900-1200 rpm using the Thermomixer R.

7.5.3 Remove the sample(s) from the Thermomixer R and allow five (5) minutes to cool to room temperature. Remove the excess liquid and dispose in a biohazardous waste container.

7.5.4 Add 30-50 µL (same volume used in section 7.5.1) of 50mM iodoacetic acid/1M ammonium bicarbonate (make from stock with high quality water) to the gel pieces.

7.5.5 Incubate the sample(s) at room temperature, in the dark, for 45-50 minutes.

7.5.6 Remove the excess liquid and dispose in a biohazardous waste container.

7.5.7 Add 100 µL of 50mM ammonium bicarbonate to the gel pieces, vortex for 10 - 15 seconds and let sit at room temperature for 5-10 minutes. Remove the excess liquid and dispose in a biohazardous waste container. Repeat two (2) more times.

7.5.8 Dehydrate the gel pieces with 100 µL of acetonitrile, vortex for 10-15 seconds and let sit at room temperature for 5-10 minutes. Remove the excess liquid and dispose in a biohazardous waste container.

7.5.9 Rehydrate the gel pieces with 100 µL of 50mM ammonium bicarbonate, vortex for 10-15 seconds and let sit at room temperature for 5-10 minutes. Remove the excess liquid and dispose in a biohazardous waste container.

7.5.10 Repeat section 7.5.8.

7.5.11 Continue the in-gel digest procedure (section 7.3) starting with section 7.3.5.

8.0 Documentation

8.1 Document all experimental conditions, reagent lot numbers, etc. on Form 22924-).

8.2 Any other pertinent information must be documented in an issued laboratory notebook or other controlled document.

8.3 All solutions must be prepared and documented as per ***SOP 22702 – Solutions Used in Process Analytics***.

9.0 References and Related Documents

9.1	**SOP 22101**	SDS-PAGE Gel Electrophoresis Using Tris-Glycine Gels
9.2	**SOP 22141**	Desalting and Buffer Exchange of Products Using illustra NAP-5 Columns
9.3	**SOP 22142**	Desalting, Buffer Exchange and Concentration of Samples Using Centrifugal Filter Devices
9.4	**SOP 22702**	Solutions Used in Process Analytics
9.5	**SOP 22910**	Operation of the Eppendorf Thermomixer R
9.6	**SOP 22916**	Operation of the Savant Automatic Environmental SpeedVac

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