Designation: E1759 – 95 (Reapproved 2003)

Standard Test Method for Isoaspartic Acid in Proteins: Method for the Determination of Asparagine Deamidation Products[1]

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INTRODUCTION

The storage of proteins in aqueous solutions often results in the formation of isoaspartic acid linkages within the polypeptide chain as a result of the deamidation of aspargine residues and the rearrangement of aspartic acid linkages. This test measures the amount of isoaspartic acid residues in a protein or peptide solution by the use of the enzyme protein isoaspartyl methyl transferase and radioactive S-adenosyl-L-methionine.

1. Scope

1.1 This test method covers the determination of isoaspartic acid residues in a protein or peptide sample. This test method is applicable for the determination of isoaspartic acid residues in a sample in the range of 2.5–50 µmol/L. Higher concentrations can be determined following dilution. The reported lower range is based on single-operator precision.

1.2 The values stated in SI units are to be regarded as the standard.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Terminology

2.1 *Definitions of Terms Specific to This Standard:*

2.1.1 *isoaspartic acid residue*—indicates an aspartic acid residue in which linkage of the polypeptide chain takes place through the gamma carboxyl group of the aspartic acid versus the alpha carboxyl group that is used in the normal peptide linkage.

3. Summary of Test Method

3.1 The basis of the procedure given in this test method is the production of radioactive methanol equal to the amount of isoaspartic acid residues present in a protein sample through the action of the enzyme protein isoaspartyl methyl transferase and radiolabelled S-adenosyl-L-methionine, a radiolabelled form of a co-factor that is consumed in the enzymatic reaction of the enzyme. During the test a radiolabelled intermediate is formed through the transfer of the labeled methyl group from S-adenosyl-L-methionine to the alpha carboxy group of isoaspartic acid. This methylated intermediate is then degraded to liberate the methyl group as methanol. The methanol is then captured in a methanol diffusion procedure and counted.

3.2 A sample of protein is incubated with the enzyme protein isoaspartyl methyl transferase and radiolabelled S-Adenosyl Methionine in a buffer that results in the accumulation of the methyl esters of isoaspartic acid residues through the enzymatic transfer of the methyl group from S-adenosyl-L-methionine to isoaspartic acid sites in the protein. The protein solution is then treated with a basic solution containing sodium dodecyl sulfate in order to inactivate the enzyme and convert the methylated isoaspartic acid residues to a succinimide and free methanol. The methanol is then separated from the protein solution through the diffusion of the methanol to a scintillation fluid solution. The methanol transferred to the scintillation fluid is then determined by counting of the radioactivity in the scintillation fluid.

4. Significance and Use

4.1 Isoaspartic acid residues are generated during incubation of proteins under a wide variety of conditions in aqueous solution. Such residues are generated most commonly through the deamidation of aspargine residues although some reports of isoaspartic acid formation through the rearrangement of aspartic acid residues have been published.

4.2 The presence of such residues can indicate that the protein containing such residues has suffered damage that may affect the biological activity of the protein. The precise

[1] This test method is under the jurisdiction of ASTM Committee E55 on Manufacture of Pharmaceutical Products and is the direct responsibility of Subcommittee E55.04 on General Biopharmaceutical Standards.

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correlation between the level of isoaspartic acid content and the biological activity of the protein needs to be determined on a case by case basis.

4.3 The test measures the level of isoaspartic acid content in a protein sample. This level will often be correlated with the degree to which the protein has suffered deamidation at asparagine residues. In addition, isoaspartic acid residues can arise on occasion through the rearrangement of aspartic acid residues. For these reasons, the level of isoaspartic acid residues in proteins can be used as a general indication that the protein sample has suffered some level of damage and should not be interpreted to indicate the precise level of damage to any one region within a protein without further testing.

5. Interfering Substances

5.1 Sodium dodecyl sulfate and guanidine hydrochloride will interfere with this test by inactivating the enzyme.

5.2 Highly acidic, basic or buffered solutions that alter the pH of the reaction mixture from pH 6.2 can interfere with the assay by altering the kinetics of the enzymatic reaction used in the test in either a positive or negative way.

6. Apparatus

6.1 *Scintillation Counter*.

6.2 *Scintillation Vials*—Scintillation vials capable of holding at least 4.5 mL of scintillation fluid and capable of being heated to 40°C for an extended period of time without damage in the presence of scintillation fluid are used.

6.3 *Microcentrifuge*.

6.4 *Positive Displacement Pipettes*.

7. Reagents and Materials

7.1 *Protein Isoaspartyl Methyltransferase.*[2]

7.2 *IsoAsp-DSIP (Delta Sleep Inducing Peptide).*[2]

7.3 *5X Reaction Buffer.*[2]

7.4 *S-Adenosyl-L-Methionine.*[2]

7.5 *Stop Solution.*[2]

7.6 *Sponge Inserts.*[2]

7.7 *Tritiated S-Adenosyl-L-Methionine, [^{3}H-SAM].*[3]

7.8 *Scintillation Cocktail*—A standard scintillation fluid with a flash point greater than or equal to 150°C and capable of use for the counting of tritiated compounds is required.

8. Calibration

8.1 Prepare 50 pmol/5 mL reference standard solution and a reaction blank solution (0 pmol/5 mL). Dilute the IsoAsp-DSIP reagent provided with the ISOQUANT[4] kit with water to create the reference standard solution and use the water for the reaction blank solution.

9. Procedure

9.1 Determine the number of reactions that will be run in the test. Each test should contain a 0 and 50 pmol IsoAsp-DSIP standard along with any unknowns. All samples and standards are to be used in duplicate specimens.

9.2 Prepare a IsoAsp-DSIP reference standard by diluting the IsoAsp-DSIP standard to 10 µmol in a 1.5 mL microcentrifuge tube with water and mixing by vortex for 15 s. Prepare at least 20 µL of diluted reference standard. Refer to the certificate of analysis provided with the IsoAsp-DSIP material for the exact concentration of the standard with the kit to be used.

9.3 Calculate the amount of ^{3}H-SAM stock solution needed in the assay. For each reaction to be run, add 1.1 µL of S-adenosyl-L-methionine and 1.1 µCi of ^{3}H-SAM to a 1.5 mL microcentrifuge tube and add water to a final volume of 11 mL.

9.4 Prepare reaction master mix. For each reaction to be run, add 11 mL of water; 11 mL of 5X reaction buffer; 11 mL of protein isoaspartyl methyltransferase; and 11 mL of ^{3}H-SAM stock solution in a 1.5 mL microcentrifuge tube. Add the materials in the order given and mix by vortex 15 s.

9.5 Place two labeled 1.5 mL microcentrifuge tubes on ice for the reaction blank, the 50 pmol IsoAsp-DSIP calibration standard and for each sample to be run.

9.6 Insert one sponge insert into a scintillation vial cap for every reaction that will be performed. Attach the sponge insert to the inside of the vial cap by removing the backing on the sponge and attaching the adhesive site of the sponge to the inside of the cap.

9.7 Fill a scintillation vial to half its capacity with scintillation fluid for each assay to be performed.

9.8 Add 10.0 mL of each unknown and reaction blank sample to the appropriate labeled sample tube and place the tube on ice. Add 5.0 mL of the IsoAsp-DSIP reference standard and 5.0 mL of water to each reference standard sample tube and place the tube on ice.

[2] Promega Corp. has an exclusive license to U.S. Patent 5 273 886 that forms the basis of this test method. All data submitted to ASTM was generated using the reagents supplied with ISOQUANT Protein Deamidation Detection Kit[4] from Promega Corp., 2800 Woods Hollow Road, Madison, WI 53711.

[3] [^{3}H]SAM ([methyl-^{3}H]-S-adenosyl-L-methionine), Amersham Cat. No. TRK614B145 (10–15 Ci/mmol) or Dupont NEN Cat. No. NET-155 (5–15 Ci/mmol), have been found satisfactory for this purpose.

[4] The ISOQUANT(TM) Protein Deamidation Detection Kit is covered by a patent held by University of California Regents, Office of Technology Transfer, 1320 Harbor Bay Parkway, Suite 150, Alameda, CA 94502. Interested parties are invited to submit information regarding the identification of acceptable alternatives to this patented item to the Committee on Standards, ASTM Headquarters. Comments will receive careful consideration at a meeting of the responsible technical subcommittee, that you may attend.