Designation: E 1759 – 95 (Reapproved 2003)

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

This standard is issued under the fixed designation E 1759; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

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 E48 on Biotechnology and is the direct responsibility of Subcommittee E48.02 on Characterization and Identification of Biological Systems.

Current edition approved Oct. 10, 1995. Published December 1995.

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.

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[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.

9.9 Every 30 s, transfer 40 mL of the reaction master mix generated in 9.4 into each labeled 1.5 mL microcentrifuge tube. Rapidly cap the tube and mix the contents for 2 to 3 s by vortex. Place the tube in a 30 ± 1°C heating block or water bath and note the time. Repeat until all reactions have been placed at 30°C.

9.10 After 30 min of incubation at 30°C, remove the first tube and place on ice. Continue removing tubes as they reach 30 min of incubation at 30°C until all tubes are again incubating on ice.

9.11 Spin the tubes at 4 ± 2°C for 10 s in a microcentrifuge to bring all liquid to the bottom of the tube. Replace the tubes on ice.

9.12 Open the first tube, add 50 mL of stop solution and mix by pipetting up and down three times.

9.13 Remove 50 mL of the mixture and apply it to the sponge insert. To apply the material properly, expel a small amount of the material from the pipettor so it forms a small drop at the tip. Press the pipettor into the sponge to half its depth and allow it to resume its original shape by removing the pressure on the pipette tip. Once the original drop of material has been absorbed into the sponge, the remaining material is gently expelled onto the sponge. The cap containing the sponge is immediately placed onto one of the scintillation vials containing scintillation fluid. Repeat 9.16 and 9.17 for each sample.

9.14 Incubate the capped vials in a 38.5 ± 1.5°C oven for 60 min.

9.15 Remove the capped vials from the oven and replace the caps containing the sponge inserts with new caps that do not contain sponge inserts. It is important that the scintillation fluid not come in contact with the sponge insert as this would result in the transfer of ^{3}H-SAM stock solution to the scintillation fluid, which would void the result.

9.16 Count the samples in a scintillation counter.

10. Calculation

10.1 Calculate the average cpm obtained per 50 pmol of the reference standard by averaging the results of the 50 pmol reference standard assays.

10.2 Calculate the number of pmoles of isoaspartic acid residues in the sample, using (Eq 1):

$$\textit{isoaspartic acid residues, pmoles} = 50 \text{ pmol} \times [S - B]/[Std - B] \quad (1)$$

where:

S = average cpm measured in the samples containing the sample,
B = average cpm measured in the reaction blanks, and
Std = average cpm calculated for 50 pmol of the reference standard obtained in 10.1.

11. Precision and Bias

11.1 The precision of this test method was measured by four laboratories at 10, 25, and 40 pmoles of isoaspartic acid DSIP in assays done in duplicate on three occasions.

11.2 Analysts using this test method with a protein solution in a solvent other than water must show the applicability of this test method to that solution composition.

12. Keywords

12.1 deamidation; isoaspartic acid residue; protein damage

APPENDIX

(Nonmandatory Information)

X1. ADDITIONAL TECHNICAL CONSIDERATIONS

X1.1 Testing Protein Samples in Complex Aqueous Solutions

X1.1.1 Testing proteins in solutions comprised of compositions other than water and protein can be done provided that the analyst has demonstrated that the materials present in the solution do not affect the test. This can be done by experimentally forming reactions containing all materials expected in the sample to be tested with the exception of protein components with concentrations of IsoAsp-DSIP ranging from 0 to 50 pmol. The reactions should be performed in duplicate at each IsoAsp-DSIP concentration and the averages from the duplicates should be calculated. The solution is judged to be acceptable in the assay if it does not cause the linearity, precision or accuracy of the assay to deviate from that obtained in reactions which do not contain the extraneous components.

X1.2 Confirmation of the Accessibility of Isoaspartic Acid Residues for a New Protein

X1.2.1 Confirmation is needed that the isoaspartic acid residues in a new protein sample are accessible to the enzyme protein isoaspartyl methyltransferase. In a few instances, the isoaspartic acid sites in a protein have become accessible to the enzyme only following digestion of the protein. In order to confirm that such sites do not exist in a new protein sample, the analyst must demonstrate that digestion of the protein does not result in an increase in measurable isoaspartic acid residues. This is done by performing the assay in parallel with digested and intact samples that are performed using the appropriate buffer and component composition for the two types of samples and demonstrating that the detected level of isoaspartic acid does not change. If an increase in the apparent

isoaspartic acid content is noted that is outside of the expected variability of the assay, the results indicate that the particular protein under consideration will need to be digested prior to the assay. Once such a determination is made, there should be no further need to confirm that the sites are accessible in each assay, though the analyst might consider occasional verification of this fact if a particular protein is being incubated under a wide variety of different conditions. A further discussion on this topic is given in the package insert provided with the ISOQUANT[4] protein deamidation detection kit.

X1.3 Confirmation of the Useful Range of the Assay for a New Protein

X1.3.1 This assay has been found to be accurate for the assay of isoaspartic acid residues in many different proteins. However, it is possible that some isoaspartic acid residues present in a new protein sample might be contained in peptide sequences that make them extremely poor substrates for the enzyme protein isoaspartyl methyltransferase. If this is the case, the measured concentration of isoaspartic acid residues in the sample might be underestimated in cases where the measured value approaches the upper useful range of the assay. The analyst can demonstrate that this effect is not operating in his system by performing the assay at two concentrations of isoaspartic acid residue for his sample, one that is in the upper range of the assay and one at a concentration one third to one half of this value. The measured values for these samples should be within the expected ratio for the dilution used taking into consideration the statistical accuracy of the assay as a whole.