Designation: D3048 – 89 (Reapproved 2009)

Standard Test Method of Assay for Alkaline Protease[1]

This standard is issued under the fixed designation D3048; 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.

1. Scope

1.1 This test method covers the assay of alkaline protease enzymes. This procedure is applicable to enzyme preparations with high activity but is inapplicable to formulated detergent products or air samples.

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this 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.* Material Safety Data Sheets are available for reagents and materials. Review them for hazards prior to usage.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D459 Terminology Relating to Soaps and Other Detergents
D1129 Terminology Relating to Water
D1193 Specification for Reagent Water
E131 Terminology Relating to Molecular Spectroscopy

3. Terminology

3.1 *Definitions:*

3.1.1 *APB unit*—that amount of enzyme which releases in 1 min under the conditions of the test a casein hydrolysate that has the same absorbance as 1 µg of tyrosine in an equivalent volume. The number of APB units per gram of a preparation is called the APB of the preparation.

3.1.2 *standardized enzyme*—an enzyme preparation of known activity for calibrating the sample enzyme in terms of a gravimetric standard of enzymatic activity.[3,4]

3.1.3 The terms "alkyl benzene sulfonate (ABS)" and "linear alkylate sulfonate (LAS)" in this method are defined in accordance with Terminologies D1129 and D459:

3.1.3.1 *alkyl benzene sulfonate (ABS)*—the generic name applied to the neutralized product resulting from the sulfonation of an alkylated benzene.

3.1.3.2 *linear alkylate sulfonate (LAS)*—a form of alkyl benzene sulfonate (ABS) in which the alkyl group is linear rather than a branched chain.

3.1.4 *nonionic surfactant*—a mixed C_{16}-C_{18} linear primary alcohol containing 65 % ethylene oxide.

3.1.5 For definitions of other terms used in these methods, refer to Terminology E131.

4. Summary of Test Method

4.1 This test is based on the hydrolysis of casein at 50°C for 15 min at pH 9. The trichloroacetic acid-soluble hydrolysate is assayed by the spectrophotometric determination of the absorbance at approximately 275 nm.[4,5] The results are correlated with the absorptivity of tyrosine or the absorbance of hydrolysate from standardized enzyme. Results are reported as APB, which is defined in Section 3, or in micrograms of pure crystalline enzyme per gram of sample.

5. Apparatus

5.1 *Water Bath*, constant-temperature, maintained at 50 ± 0.2°C.

5.2 *Ultraviolet Spectrophotometer*, suitable for liquid measurements at a wavelength of approximately 275 nm.

5.3 *Absorption Cell*, silica, 10-mm light path.

5.4 *pH Meter*.

5.5 *Test Tubes*, 25 by 150 mm.

[1] This test method is under the jurisdiction of ASTM Committee D12 on Soaps and Other Detergents and is the direct responsibility of Subcommittee D12.12 on Analysis and Specifications of Soaps, Synthetics, Detergents and their Components.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The sole source of supply known to the committee at this time is National Institute of Occupational Safety and Health, 1014 Broadway Ave., Cincinnati, Ohio 45202.

[4] If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[5] Bailey, J. L. "Techniques in Protein Chemistry," Elsevier Publishing Co., New York, NY. Chapter 11, 1967, pp. 340–352.

6. Reagents

6.1 *Purity of Reagent*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.[6] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

6.2 Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193.

6.3 *Acetic Acid* (6.67 *M*)—Mix 400 g of glacial acetic acid (CH_3COOH) with sufficient water to yield 1 L of solution.

6.4 *Borate Buffer* (0.2 *M*)—Dissolve 12.4 g of boric acid (H_3BO_3) in 100 mL of 1 *N* NaOH solution and dilute to 1 L with water.

6.5 *Enzyme Buffer Solution*—Dissolve 12.0 g of sodium chloride (NaCl) in about 500 mL of water and add 237 mL of 0.2 M borate buffer. Adjust to pH 9.0 with 0.1 *N* NaOH solution. Approximately 18 mL will be needed. Dilute to 1 L with water.

6.6 *Enzyme Media*—Combine equal volumes of substrate (6.9) and synthetic detergent base (6.10) in sufficient quantity to accommodate each sample and thermally equilibrate this solution at 50°C. Each analysis requires 20 mL of this solution, 10 for assay and 10 for the control; samples should be run in triplicate.

6.7 *Sodium Hydroxide, Standard Solution (1 N)*—Dissolve 40 g of sodium hydroxide solution (NaOH) in water and dilute to 1 L. For a 0.1 *N* solution, dilute 100 mL of 1 *N* NaOH solution to 1 L with water.

6.8 *Standardized Enzyme*—Dissolve 100 mg of the standardized enzyme preparation[4,3] (3.1.2) in enzyme buffer solution (6.5), and dilute to 100 mL with the same solution.

6.9 *Substrate*—Slurry 6.0 g (dry basis) of casein[4,7] in 200 mL of water, add 120 mL of 0.2 *M* borate buffer, and heat 20 min in a boiling water bath. Cool to room temperature and adjust to pH 9.0 with 0.1 *N* NaOH solution. About 30 mL will be needed. Check the pH at higher dilution before adjusting with water to a final volume of 500 mL. This solution is stable for 1 week but should be stored under refrigeration.

6.10 *Synthetic Detergent Base*—Dissolve 0.3 g of nonionic surfactant[4,8] (3.1.4) in 350 mL of water at 50°C by stirring. Add 0.30 g of LAS[4,9] (3.1.3) and 1.5 g of sodium tripolyphosphate. Stir to dissolve, and adjust to pH 9.0 with about 6 mL of 0.1 *N* HCl. Dilute to 500 mL with water.

6.11 *Trichloroacetic Acid* (*TCA*) *Solution*—Dissolve 20.45 g of TCA (CCl_3COOH) and 21.6 g of crystalline sodium acetate trihydrate ($CH_3COONa{\cdot}3H_2O$) in about 300 mL of water. Add 56.9 mL of 6.67 *M* CH_3COOH and dilute to 1 L with water. This solution is unstable and should be discarded after 1 week.

6.12 *Tyrosine Standard*—Dissolve 100 mg of L-tyrosine, previously dried in a desiccator, in 60 mL of 0.1 *N* HCl. Upon complete dissolution of the tyrosine dilute to 1 L with water.

7. Safety Precautions

7.1 Avoid generating or breathing enzyme dust.

8. Assay

8.1 *Sample*—Prepare all solutions and serial dilutions of the sample with enzyme buffer solution (6.5). Stock solutions should be stirred for 30 min before serial dilutions are made and may be held for a maximum of 8 h. Use at least a 100 mg of the sample enzyme preparation for the initial stock solution. The final or working sample solution should contain 0.030 to 0.060 mg/mL of solution. An activity of approximately 600 000 APB or an absorbance of approximately 0.6 should be observed for the 5-mL aliquot of sample solution used in this assay.

8.2 *Digestion*—Triplicate analyses of samples and con-trols are recommended.

8.2.1 *Sample*—Thermally equilibrate 5-mL aliquots of sample solution (8.1) in 25 by 150-mm tubes. Add 10 mL of enzyme media (6.6) and digest for 15 min in a water bath at 50°C. Add 10 mL of TCA reagent (6.11), shake vigorously, and retain at 50°C for 30 min with intermittent shaking.

8.2.2 *Control*—Incubate 10 mL of enzyme media (6.6) and approximately 5 mL of sample solution (8.1) for 15 min at 50°C in separate tubes. Add 10 mL of TCA reagent (6.11) to the 10-mL aliquot of enzyme media, shake, and hold 1 min. Add 5 mL of the incubated sample solution, shake vigorously, and hold for 30 min as above.

8.3 *Removal of Precipitate*:

8.3.1 *Centrifugation*—The precipitated mixtures (8.2.1 and 8.2.2) can be clarified by centrifugation.

8.3.2 *Filtration*—Filter[4,10] the precipitated mixture after thorough shaking. Refilter the first portion of the filtrate through the same filter for a clear filtrate.

8.4 *Absorbance Measurement*—Determine the absorbance of the supernatant in a 10-mm cell at 275 nm. Set the instrument at zero absorbance with the incubated control solution (8.2.2).

9. Calibration

9.1 *Tyrosine Standard*—Prepare serial dilutions of the tyrosine standard (6.12) and determine the absorbance of each at 275 nm using a 10-mm cell path. Use a water blank to adjust

[6] *Reagent Chemicals, American Chemical Society Specifications* , American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[7] The sole source of supply of Hammersten casein known to the committee at this time is Nutritional Biochemical Corp. Cleveland, Ohio 44128.

[8] The sole source of supply of the Alfol 1618–65 known to the committee at this time is Continental Oil Co., Ponca City, OK 74601.

[9] The sole source of supply of Sulframin 1345 known to the committee at this time is Witco Chemical Corp., New York, NY. 1001s has been found suitable. The latter material is only 82.6 percent active and a suitable increase must be made. LAS reference material may be obtained from the Soap & Detergent Assn., 485 Madison Ave., New York, NY 10022.

[10] The sole source of supply of the Whatman No. 42 filter paper known to the committee at this time is Sargent-Welch, Skokie, IL 60077 has been found suitable for this purpose.

the instrument at zero absorbance. Prepare a graph of absorbance *versus* µg/mL of tyrosine for the range 25 to 100 µg/mL. A straight line passing through the origin must be obtained. An absorptivity value of approximately 0.0072 mL/(µg cm) should result.

$$a_T = A/bc \quad (1)$$

where:
a_T = absorptivity value of tyrosine,
A = absorbance of sample,
b = cell path, 1 cm, and
c = concentration, µg/mL of tyrosine.

9.2 *Standardized Enzyme*—Prepare serial dilutions of standardized enzyme (6.8) with enzyme buffer solution (6.5). Perform the digestion using the standardized enzyme solutions as samples as described for sample and control (8.2.1 and 8.2.2). Prepare a graph of the absorbance of TCA-soluble hydrolysate *versus* serial dilution of standardized enzyme for concentrations yielding absorbances of 0.2 to 0.8.

10. Calculations

10.1 *APB Units*—Calculate the number of APB units (3.1) as follows:

$$\text{APB units} = (V/t \times (A_e/a_T) \quad (2)$$

where:
V = total volume of enzyme hydrolysate, 25 mL in this procedure,
t = total digestion time in min, 15 min in this procedure,
A_e = absorbance of enzyme hydrolysate *versus* control, and
a_T = absorptivity value of tyrosine in mL/(µg·cm).

10.2 *APB*—Calculate the number of APB units per gram of sample, APB, (3.1) as follows:

$$\text{APB} = \text{APB units}/W \quad (3)$$

where:
W = grams of enzyme preparation used in the assay. In this procedure the value is grams per 5 mL of sample solution.

10.3 *Standard Enzyme*—Calculate the concentration of enzyme in the sample, µg/g sample, as follows:

$$\text{Concentration, µg/g} = S/W \quad (4)$$

where:
S = micrograms of standardized enzyme interpolated from the standard curve (9.2).

11. Keywords

11.1 assay; alkaline protease; enzyme