Viebrock, Lauren

From: 岡戸信夫 <nokado@snc-enzymes.co.jp>
Sent: Friday, May 14, 2021 12:59 AM
To: Viebrock, Lauren
Subject: [EXTERNAL] Re: GRN 000965 Questions
Attachments: GRN 965-Response to FDA Questions_May14_snc.pdf

Dear Lauren,

Thank you very much for reviewing the GRAS Notice No. 000965, as well as your questions.

Please find the attachment for our responses to your questions.

Should you have further questions, please do not hesitate to reach out to me.

Yours sincerely,

++

Nobuo Okado

Quality Assurance

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Mail; nokado@snc-enzymes.co.jp

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2021年5月1日(土) 2:36 Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>:

> Dear Mr. Okado,
>
> During our review of GRAS Notice No. 000965, we noted questions that need to be addressed. Please find the questions attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,

Lauren

Lauren VieBrock
Regulatory Review Scientist/Microbiology Reviewer

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Responses to the U.S. FDA's Questions Related to GRAS Notice No. GRN 000965

In response to the United States Food and Drug Administration's questions (dated 30 April 2021) pertaining to GRAS Notice No. GRN 000965, as submitted by Shin Nihon for arabinase enzyme preparation produced by *Aspergillus tubingensis*, below please find the additionally requested information.

Question 1

Please clarify if wheat bran, an ingredient in the fermentation medium, is removed from the final enzyme preparation.

Response to Question 1

Yes, the wheat bran, which is used as an ingredient in the fermentation medium, is removed from the final enzyme preparation.

The wheat bran provides a source of nutrition for the production organism during the fermentation. The wheat bran is utilized by the microorganism, with a progressive decrease in levels during the course of the fermentation. After fermentation, produced arabinase is extracted from the fermented medium with water. Wheat bran remains in the fermented medium.
In order to confirm the removal of wheat bran, samples of both the final arabinase enzyme concentrate and the finished (formulated) enzyme preparation were tested for presence of residual levels of protein, including gluten, and gliadin specifically, as derived from wheat bran. The testing was performed using enzyme-linked immunosorbent assay (ELISA)-based methods (limits of quantification: 1 ppm). As presented in Table 1, no quantifiable levels of wheat protein residues were identified in any of the tested samples.

Table 1 Levels of Wheat Protein/Gluten in Samples of Arabinase Concentrate and Arabinase Final Preparation Obtained using *Aspergillus tubingensis* GPA41

Samples (Lot No.)	Levels of Wheat Protein and/or Gluten (Gliadin) (ppm)	
	ELISA Method 1[a]	ELISA Method 2[b]
Enzyme Concentrate		
190313T4	<1	<1
191206T1	<1	<1
210129T4	<1	<1
Final Formulated Product		
200128-08	<1	<1
210402-01	<1	<1
200220-01	<1	<1
210421-10	<1	<1

ELISA = enzyme-linked immunosorbent assay; ppm = parts per million.
[a] ELISA Kit II (Morinaga Institute of Biological Science, Inc.) – tests for gliadin specifically.
[b] FASTKIT ELISA ver. III (NIHON HAM) – tests for whole wheat protein, including gluten.

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Question 2

Please provide an updated reference for FCC 11th Edition.

Response to Question 2

The references from the 11th Edition of the Food Chemicals Codex (FCC, 2019a; FCC, 2019b) are replaced by the 12th Edition. The updated references are provided in Appendix A. In summary:

> FCC (2021a). Enzyme preparations. In: *Food Chemicals Codex, 12th edition.* (Suppl. 1). Rockville (MD): United States Pharmacopeial Convention.
>
> replaces:
>
> FCC (2019a). Enzyme preparations. In: *Food Chemicals Codex, 11th edition, 2nd Supplement*. Rockville (MD): United States Pharmacopeia Convention (USP).
>
> and
>
> FCC (2021b). Appendix V. Enzyme preparations used in food processing. In: *Food Chemicals Codex, 12th edition.* (Suppl. 2). Rockville (MD): United States Pharmacopeial Convention.
>
> replaces:
>
> FCC (2019b). Appendix V: Enzyme preparations used in food processing. In: *Food Chemicals Codex, 11th edition, 2nd Supplement*. Rockville (MD): United States Pharmacopeia Convention (USP).

Question 3

Please provide a narrative to clarify the statement on Page 67 of the notice, "A large margin of safety exists between the NOAEL and the estimated level of exposure to Sumizyme AG from foods (1.35 mg TOS/kg body weight/day)".

Response to Question 3

The results of the 90-day toxicity study in which the ultra-filtered concentrate of arabinase was administered to male and female rats by gavage at doses providing 0, 15, 153, or 1,530 mg total organic solids (TOS)/kg body weight/day are summarized in Part 6, Section 6.3.2.1 of the GRAS Notice (pp. 33-34). Under the conditions of this study, the highest dose tested of 1,530 mg TOS/kg body weight/day was determined to be the no-observed-adverse-effect level (NOAEL) for the arabinase concentrate.

As presented in Part 3, Section 3.2 of the GRAS Notice (p. 27), a theoretical maximum daily intake (TMDI) of 1.35 mg TOS/kg body weight/day was calculated for the arabinase enzyme preparation under the intended conditions of use and considering a number of conservative assumptions.

Therefore, in comparison to the human exposure estimate of 1.35 mg TOS/kg body weight/day, the NOAEL of 1,530 mg TOS/kg body weight/day in the 90-day study is 1,133-fold greater (a margin of safety of ≥1,000 exists between the intake estimated for the arabinase enzyme preparation under proposed conditions, and the NOAEL derived from the 90-day study).

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Appendix A
Updated References from the Food Chemicals Codex, 12th Edition (2021)

Enzyme Preparations

Enzyme Preparations

Published in: FCC 12 1S | FCC 12 2S
First Published: Prior to FCC 6
Last Revised: FCC 9, Second Supplement

DESCRIPTION

Enzyme Preparations used in food processing are derived from animal, plant, or microbial sources (see *Classification*, below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one active component or, more commonly, a mixture of several, as well as food-grade diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practices. The individual preparations usually are named according to the substance to which they are applied, such as *Protease* or *Amylase*. Traditional names such as *Malt, Pepsin*, and *Rennet* also are used, however. The color of the preparations—which may be liquid, semiliquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand. The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for *Food Chemicals Codex* purposes), the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food to achieve the desired effect. Additional information relating to the nomenclature and the sources from which the active components are derived is provided under *Appendix V: Enzyme Assays*.

FUNCTION: Enzyme (see discussion under *Classification*, below)

PACKAGING AND STORAGE: Store in well-closed containers in a cool, dry place.

IDENTIFICATION

CLASSIFICATION

- **ANIMAL-DERIVED PREPARATIONS**

Catalase, Bovine Liver: Produced as partially purified liquid or powdered extracts from bovine liver. Major active principle: *catalase*. Typical application: used in the manufacture of certain cheeses.

Chymotrypsin: Obtained from purified extracts of bovine or porcine pancreatic tissue. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymotrypsin*. Typical application: used in the hydrolysis of protein.

Lipase, Animal: Obtained from the edible forestomach tissue of calves, kids, or lambs; and from animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts dispersible in water, but insoluble in alcohol. Major active principle: *lipase*. Typical applications: used in the manufacture of cheese and in the modification of lipids.

Lysozyme: Obtained from extracts of purified chicken egg whites. Generally prepared and used in the hydrochloride form as a white powder. Major active principle: *lysozyme*. Typical application: used as an antimicrobial in food processing.

Pancreatin: Obtained from porcine or bovine (ox) pancreatic tissue. Produced as a white to tan, water-soluble powder. Major active principles: (1) α-amylase; (2) protease; and (3) lipase. Typical applications: used in the preparation of precooked cereals, infant foods, and protein hydrolysates.

Pepsin: Obtained from the glandular layer of hog stomach. Produced as a white to light tan, water-soluble powder; amber paste; or clear, amber to brown, aqueous liquids. Major active principle: *pepsin*. Typical applications: used in the preparation of fishmeal and other protein hydrolysates and in the clotting of milk in the manufacture of cheese (in combination with rennet).

Phospholipase A_2: Obtained from porcine pancreatic tissue. Produced as a white to tan powder or pale to dark yellow liquid. Major active principle: *phospholipase* A_2. Typical application: used in the hydrolysis of lecithins.

Rennet, Bovine: Aqueous extracts made from the fourth stomach of bovines. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (pepsin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of sheep or goats.

Rennet, Calf: Aqueous extracts made from the fourth stomach of calves. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (chymosin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of lambs or kids.

Trypsin: Obtained from purified extracts of porcine or bovine pancreas. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *trypsin*. Typical applications: used in baking, in the tenderizing of meat, and in the production of protein hydrolysates.

- **PLANT-DERIVED PREPARATIONS**

Amylase: Obtained from extraction of ungerminated barley or extraction from grains of wheat. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: β-*amylase*. Typical applications: used in the production of alcoholic beverages and sugar syrups.

Bromelain: The purified proteolytic substance derived from the pineapples *Ananas comosus* and *Ananas bracteatus* L. (Fam. Bromeliaceae). Produced as a white to light tan, amorphous powder soluble in water (the solution is usually colorless to light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *bromelain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, in the production of protein hydrolysates, and in baking.

Ficin: The purified proteolytic substance derived from the latex of *Ficus* sp. (Fam. Moraceae), which includes a variety of tropical fig trees. Produced as a white to off-white powder completely soluble in water. (Liquid fig latex concentrates are light to dark brown.) Major active principle: *ficin*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, and in the conditioning of dough in baking.

Malt: The product of the controlled germination of barley. Produced as a clear amber to dark brown liquid preparation or as a white to tan powder. Major active principles: (1) α-*amylase* and (2) β-*amylase*. Typical applications: used in baking, in the manufacture of alcoholic beverages and of syrups.

Papain: The purified proteolytic substance derived from the fruit of the papaya *Carica papaya* L. (Fam. Caricaceae). Produced as a white to light tan, amorphous powder or a liquid soluble in

water (the solution is usually colorless or light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *papain* and (2) *chymopapain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, and in the production of protein hydrolysates.

- **MICROBIALLY-DERIVED PREPARATIONS**

α-Acetolactatedecarboxylase: (*Bacillus subtilis* containing a *Bacillus brevis* gene) Produced as a brown liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually a light yellow to brown). Major active principle: *decarboxylase*. Typical application: used in the preparation of beer.

Aminopeptidase, Leucine: (*Aspergillus niger* var., *Aspergillus oryzae* var., and other microbial species) Produced as a light tan to brown powder or as a brown liquid by controlled fermentation using *Aspergillus niger* var., *Aspergillus oryzae* var., or other microbial species. The powder is soluble in water (the solution is usually light yellow to brown). Major active principles: (1) *aminopeptidase*, (2) *protease*, and (3) *carboxypeptidase* activities in varying amounts. Typical applications: used in the preparation of protein hydrolysates and in the development of flavors in processed foods.

Carbohydrase: (*Aspergillus niger* var., including *Aspergillus aculeatus*) Produced as an off-white to tan powder or a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. (including *Aspergillus aculeatus*). Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase* (a mixture of enzymes, including *pectin depolymerase, pectin methyl esterase, pectin lyase*, and *pectate lyase*), (3) *cellulase*, (4) *glucoamylase* (amyloglucosidase), (5) *amylo-1,6-glucosidase*, (6) *hemicellulase* (a mixture of enzymes, including *poly(galacturonate) hydrolase, arabinosidase, mannosidase, mannanase*, and *xylanase*), (7) *lactase*, (8) β-*glucanase*, (9) β-D-*glucosidase*, (10) *pentosanase*, and (11) α-*galactosidase*. Typical applications: used in the preparation of starch syrups and dextrose, alcohol, beer, ale, fruit juices, chocolate syrups, bakery products, liquid coffee, wine, dairy products, cereals, and spice and flavor extracts.

Carbohydrase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *glucoamylase* (amyloglucosidase), and (3) *lactase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, ale, bakery products, and dairy products.

Carbohydrase: (*Bacillus acidopullulyticus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Bacillus acidopullulyticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *pullulanase*. Typical applications: used in the hydrolysis of amylopectins and other branched polysaccharides.

Carbohydrase: (*Bacillus stearothermophilus*) Produced as an off-white to tan powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus stearothermophilus*. Soluble in water, but practically insoluble in alcohol, in ether, and in chloroform. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, and bakery products.

Carbohydrase: (*Candida pseudotropicalis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Candida pseudotropicalis*. Soluble in water (the solution is usually light yellow to dark brown) but insoluble in alcohol, in chloroform, and

in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Kluyveromyces marxianus* var. *lactis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Kluyveromyces marxianus* var. *lactis*. Soluble in water (the solution is usually light yellow to dark brown), but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Mortierella vinaceae* var. *raffinoseutilizer*) Produced as an off-white to tan powder or as pellets by controlled fermentation using *Mortierella vinaceae* var. *raffinoseutilizer*. Soluble in water (pellets may be insoluble in water), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*galactosidase*. Typical application: used in the production of sugar from sugar beets.

Carbohydrase: (*Rhizopus niveus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Rhizopus niveus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *glucoamylase*. Typical application: used in the hydrolysis of starch.

Carbohydrase: (*Rhizopus oryzae* var.) Produced as a powder or a liquid by controlled fermentation using *Rhizopus oryzae* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase*, and (3) *glucoamylase* (amyloglucosidase). Typical applications: used in the preparation of starch syrups and fruit juices, vegetable purees, and juices and in the manufacture of cheese.

Carbohydrase: (*Saccharomyces* species) Produced as a white to tan, amorphous powder by controlled fermentation using a number of species of *Saccharomyces* traditionally used in the manufacture of food. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *invertase* and (2) *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: [(*Trichoderma longibrachiatum* var.) (formerly *reesei*)] Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Trichoderma longibrachiatum* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *cellulase*, (2) β-*glucanase*, (3) β-D-*glucosidase*, (4) *hemicellulase*, and (5) *pentosanase*. Typical applications: used in the preparation of fruit juices, wine, vegetable oils, beer, and baked goods.

Carbohydrase: (*Bacillus subtilis* containing a *Bacillus megaterium* α-*amylase* gene) Produced as an off-white to brown, amorphous powder or liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, and dextrose.

Carbohydrase: (*Bacillus subtilis* containing a *Bacillus stearothermophilus* α-amylase gene) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: maltogenic *amylase*. Typical applications: used in the preparation of starch syrups, dextrose, alcohol, beer, and baked goods.

Carbohydrase and Protease, Mixed: (*Bacillus licheniformis* var.) Produced as an off-white to brown, amorphous powder or as a liquid by controlled fermentation using *Bacillus licheniformis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *protease*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, fishmeal, and protein hydrolysates.

Carbohydrase and Protease, Mixed: (*Bacillus subtilis* var. including *Bacillus amyloliquefaciens*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Bacillus subtilis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) β-*glucanase*, (3) *protease*, and (4) *pentosanase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, bakery products, and fishmeal, in the tenderizing of meat, and in the preparation of protein hydrolysates.

Catalase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical applications: used in the manufacture of cheese, egg products, and soft drinks.

Catalase: (*Micrococcus lysodeikticus*) Produced by controlled fermentation using *Micrococcus lysodeikticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical application: used in the manufacture of cheese, egg products, and soft drinks.

Chymosin: (*Aspergillus niger* var. *awamori, Escherichia coli K-12*, and *Kluyveromyces marxianus*, each microorganism containing a calf *prochymosin* gene) Produced as a white to tan, amorphous powder or as a light yellow to brown liquid by controlled fermentation using the above-named genetically modified microorganisms. The powder is soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymosin*. Typical application: used in the manufacture of cheese and in the preparation of milk-based desserts.

Glucose Isomerase: (*Actinoplanes missouriensis, Bacillus coagulans, Streptomyces olivaceus, Streptomyces olivochromogenes, Microbacterium arborescens, Streptomyces rubiginosus* var., or *Streptomyces murinus*) Produced as an off-white to tan, brown, or pink amorphous powder, granules, or liquid by controlled fermentation using any of the above-named organisms. The products may be soluble in water, but practically insoluble in alcohol, in chloroform, and in ether; or if immobilized, may be insoluble in water and partially soluble in alcohol, in chloroform, and in ether. Major active principle: *glucose* (or *xylose*) *isomerase*. Typical applications: used in the manufacture of high-fructose corn syrup and other fructose starch syrups.

Glucose Oxidase: (*Aspergillus niger* var.) Produced as a yellow to brown solution or as a yellow to tan or off-white powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *glucose oxidase* and (2) *catalase*. Typical applications: used in the removal of sugar from liquid eggs and in the deoxygenation of citrus beverages.

Lipase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active

principle: *lipase*. Typical application: used in the hydrolysis of lipids (e.g., fish oil concentrates and cereal-derived lipids).

Lipase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids (e.g., fish oil concentrates) and in the manufacture of cheese and cheese flavors.

Lipase: (*Candida rugosa;* formerly *Candida cylindracea*) Produced as an off-white to tan powder by controlled fermentation using *Candida rugosa*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of dairy products and confectionery goods, and in the development of flavor in processed foods.

Lipase: [*Rhizomucor (Mucor) miehei*] Produced as an off-white to tan powder or as a liquid by controlled fermentation using *Rhizomucor miehei*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of cheese, and in the removal of haze in fruit juices.

Phytase: (*Aspergillus niger* var.) Produced as an off-white to brown powder or as a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *3-phytase* and (2) *acid phosphatase*. Typical applications: used in the production of soy protein isolate and in the removal of phytic acid from plant materials.

Protease: (*Aspergillus niger* var.) Produced by controlled fermentation using *Aspergillus niger* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the production of protein hydrolysates.

Protease: (*Aspergillus oryzae* var.) Produced by controlled fermentation using *Aspergillus oryzae* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical applications: used in the chillproofing of beer, in the production of bakery products, in the tenderizing of meat, in the production of protein hydrolysates, and in the development of flavor in processed foods.

Rennet, Microbial: (nonpathogenic strain of *Bacillus cereus*) Produced as a white to tan, amorphous powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus cereus*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: (*Endothia parasitica*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using nonpathogenic strains of *Endothia parasitica*. The powder is soluble in water (the solution is usually tan to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: [*Rhizomucor (Mucor)* sp.] Produced as a white to tan, amorphous powder by controlled fermentation using *Rhizomucor miehei*, or *pusillus* var. Lindt. The powder is soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Transglutaminase: (*Streptoverticillium mobaraense* var.) Produced as an off-white to weak yellow-brown, amorphous powder by controlled fermentation using *Streptoverticillium mobaraense* var. Soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *transglutaminase*. Typical applications: used in the processing of meat, poultry, and seafood; production of yogurt, certain cheeses, and frozen desserts; and manufacture of pasta products and noodles, baked goods, meat analogs, ready-to-eat cereals, and other grain-based foods.

- **Reactions Catalyzed**

[Note—The reactions catalyzed by any given active component are essentially the same, regardless of the source from which that component is derived.]

α-Acetolactatedecarboxylase: Decarboxylation of α-cetolactate to acetoin

Aminopeptidase, Leucine: Hydrolysis of *N*-terminal amino acid, which is preferably leucine, but may be other amino acids, from proteins and oligopeptides, yielding free amino acids and oligopeptides of lower molecular weight

α-Amylase: Endohydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides

β-Amylase: Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding maltose and betalimit dextrins

Bromelain: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

Catalase: $2H_2O_2 \leftrightarrow O_2 + 2H_2O$

Cellulase: Hydrolysis of β-1,4-glucan bonds in such polysaccharides as cellulose, yielding β-dextrins

Chymosin (calf and fermentation derived): Cleaves a single bond in kappa casein

Ficin: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

α-Galactosidase: Hydrolysis of terminal nonreducing α-D-galactose residues in α-D-galactosides

β-Glucanase: Hydrolysis of β-1,3- and β-1,4-linkages in β-D-glucans, yielding oligosaccharides and glucose

Glucoamylase (amyloglucosidase): Hydrolysis of terminal α-1,4- and α-1,6-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding glucose (dextrose)

Glucose Isomerase (xylose isomerase): Isomerization of glucose to fructose, and xylose to xylulose

Glucose Oxidase: β-D-glucose + $O_2 \leftrightarrow$ D-glucono-δ-lactone + H_2O_2

β-D-Glucosidase: Hydrolysis of terminal, nonreducing β-D-glucose residues with the release of β-D-glucose

Hemicellulase: Hydrolysis of β-1,4-glucans, α-L-arabinosides, β-D-mannosides, 1,3-β-D-xylans, and other polysaccharides, yielding polysaccharides of lower molecular weight

Invertase (β-fructofuranosidase): Hydrolysis of sucrose to a mixture of glucose and fructose (invert sugar)

Lactase (β-galactosidase): Hydrolysis of lactose to a mixture of glucose and galactose

Lysozyme: Hydrolysis of cell-wall polysaccharides of various bacterial species leading to the breakdown of the cell wall most often in Gram-positive bacteria

Maltogenic Amylase: Hydrolysis of α-1,4-glucan bonds

Lipase: Hydrolysis of triglycerides of simple fatty acids, yielding mono- and diglycerides, glycerol, and free fatty acids

Pancreatin

α-Amylase: Hydrolysis of α-1,4-glucan bonds

Protease: Hydrolysis of proteins and polypepticles

Lipase: Hydrolysis of triglycerides of simple fatty acids

Pectinase

Pectate lyase: Hydrolysis of pectate to oligosaccharides

Pectin depolymerase: Hydrolysis of 1,4 galacturonide bonds

Pectin lyase: Hydrolysis of oligosaccharides formed by pectate lyase

Pectinesterase: Demethylation of pectin

Pepsin: Hydrolysis of polypeptides, including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues, yielding peptides of lower molecular weight

Phospholipase A_2: Hydrolysis of lecithins and phosphatidylcholine, producing fatty acid anions

Phytase

3-Phytase: *myo*-Inositol hexakisphosphate + H_2O↔1,2,4,5,6-pentakisphosphate + orthophosphate

Acid Phosphatase: Orthophosphate monoester + H_2O↔an alcohol + orthophosphate

Protease (generic): Hydrolysis of polypeptides, yielding peptides of lower molecular weight

Pullulanase: Hydrolysis of 1,6-α-D-glycosidic bonds on amylopectin and glycogen and in α- and β-limit dextrins, yielding linear polysaccharides

Rennet (bovine and calf): Hydrolysis of polypeptides; specificity may be similar to pepsin

Transglutaminase: Binding of proteins

Trypsin: Hydrolysis of polypeptides, amides, and esters at bonds involving the carboxyl groups of L-arginine and L-lysine, yielding peptides of lower molecular weight

ASSAY

- **Procedure**

Analysis: The following procedures, which are included under *Appendix V: Enzyme Assays*, are provided for application as necessary in determining compliance with the declared representations for enzyme activity[1]: α-Acetolactatedecarboxylase Activity, Acid Phosphatase Activity, α-Amylase Activity (Nonbacterial); Bacterial α-Amylase Activity (BAU); Catalase Activity; Cellulase Activity; Chymotrypsin Activity; Diastase Activity (Diastatic Power); α-Galactosidase Activity, β-Glucanase Activity; Glucoamylase Activity (Amyloglucosidase Activity); Glucose Isomerase Activity; Glucose Oxidase Activity; β-D-Glucosidase Activity; Hemicellulase Activity; Invertase Activity; Lactase (Neutral) (β-Galactosidase) Activity; Lactase (Acid) (β-Galactosidase) Activity; Lipase Activity; Lipase/Esterase (Forestomach) Activity; Maltogenic Amylase Activity; Milk-Clotting Activity; Pancreatin Activity; Pepsin Activity; Phospholipase Activity; Phytase Activity; Plant Proteolytic Activity; Proteolytic Activity, Bacterial (PC); Proteolytic Activity, Fungal (HUT); Proteolytic Activity, Fungal (SAP); Pullulanase Activity; and Trypsin Activity.

Acceptance criteria: NLT 85.0% and NMT 115.0% of the declared units of enzyme activity

IMPURITIES

- **Lead, *Lead Limit Test, Appendix IIIB***

Control: 5 µg Pb (5 mL of *Diluted Standard Lead Solution*)

Acceptance criteria: NMT 5 mg/kg

SPECIFIC TESTS

- **Microbial Limits**

[Note—Current methods for the following tests may be found in the Food and Drug Administration's Bacteriological Analytical Manual online at *www.fda.gov/Food/default.htm*.]

Acceptance criteria

Coliforms: NMT 30 CFU/g

Salmonella: Negative in 25 g

OTHER REQUIREMENTS

Enzyme preparations are produced in accordance with good manufacturing practices. Regardless of the source of derivation, they should cause no increase in the total microbial count in the treated food over the level accepted for the respective food.

Animal tissues used to produce enzymes must comply with the applicable U.S. meat inspection requirements and must be handled in accordance with good hygienic practices.

Plant material used to produce enzymes or culture media used to grow microorganisms consist of components that leave no residues harmful to health in the finished food under normal conditions of use.

Preparations derived from microbial sources shall be obtained using a pure culture fermentation of a non-pathogenic and non-toxigenic strain and are produced by methods and under culture conditions that ensure a controlled fermentation, thus preventing the introduction of microorganisms that could be the source of toxic materials and other undesirable substances.

The carriers, diluents, and processing aids used to produce the enzyme preparations shall be substances that are acceptable for general use in foods, including water and substances that are insoluble in foods but removed from the foods after processing.

Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.

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[1] Because of the varied conditions under which pectinases are employed, and because laboratory hydrolysis of a purified pectin substrate does not correlate with results observed with the natural substrates under use conditions, pectinase suppliers and users should develop their own assay procedures that would relate to the specific application under consideration.

Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
ENZYME PREPARATIONS	Tongtong Xu Associate Scientific Liaison +1 (301) 692-3659 Ext.3659	FI2020 Food Ingredients

Page Information

- FCC 12 - page 406
- FCC 12 - page 405
- FCC 12 - page 404

APPENDIX V: ENZYME ASSAYS

APPENDIX V: ENZYME ASSAYS

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ENZYME PREPARATIONS USED IN FOOD PROCESSING

A list of the enzymes covered by the general monograph on *Enzyme Preparations* is shown in *Table 1*. Also incorporated in the table are the trivial names by which each is commonly known, as well as the systematic names of the major components or of the enzyme for which the preparation is standardized, in accordance with the *Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes*.

Table 1. Enzyme Preparations Used in Food Processing

TRIVIAL NAME	CLASSIFICATION	SOURCE	NAMES (IUB)[a]
α-Acetolactatedecarboxylase	Lyase	*Bacillus subtilis*[*]	(2*S*)-2-Hydroxy-2-methyl-3-oxobutanoate carboxy-lyase[(3*R*)-3-hydroxybutan-2-one-forming]
Aminopeptidase, Leucine	Protease	(1) *Aspergillus niger*	None
		(2) *Aspergillus oryzae*	
		(3) *Rhizopus oryzae*	
		(4) *Rhizopus arrhizus*	
		(5) *Lactococcus lactis*	
		(6) *Trichoderma reesei*	
		(7) *Trichoderma*	

TRIVIAL NAME	CLASSIFICATION	SOURCE	NAMES (IUB)[a]
		longibrachiatum	
		(8) *Aeromonas caviae*	
		(9) *Lactobacillus casei*	
α-Amylase	Carbohydrase	(1) *Aspergillus niger* var.	1,4-α-D-Glucan glucanohydrolase
		(2) *Aspergillus oryzae* var.	
		(3) *Rhizopus oryzae* var.	
		(4) *Bacillus subtilis* var.	
		(5) Barley malt	
		(6) *Bacillus licheniformis* var.	
		(7) *Bacillus stearothermophilus*	
		(8) *Bacillus subtilis** (d-*Bacillus megaterium*)	
		(9) *Bacillus subtilis** (d-*Bacillus stearothermophilus*)	
		(10) *Bacillus licheniformis** (d-*Bacillus stearothermophilus*)	
β-Amylase	Carbohydrase	(1) Barley malt	1,4-α-D-Glucan maltohydrolase
		(2) Barley	
		(3) Wheat	
Bromelain	Protease	Pineapples: *Ananas comosus Ananas bracteatus* (L)	None

TRIVIAL NAME	CLASSIFICATION	SOURCE	NAMES (IUB)[a]
Catalase	Oxidoreductase	(1) *Aspergillus niger* var.	Hydrogen peroxide: hydrogen peroxide oxidoreductase
		(2) Bovine liver	
		(3) *Micrococcus lysodeikticus*	
Cellulase	Carbohydrase	(1) *Aspergillus niger* var.	*endo*-1,4-(1,3;1,4)-β-D-Glucan 4-glucanohydrolase
		(2) *Trichoderma longibrachiatum* (formerly *reesei*)	
Chymosin	Protease	(1) *Aspergillus niger* var. *awamori** (d-calf prochymosin gene)	
		(2) *Escherichia coli* K-12* (d-calf prochymosin gene)	
		(3) *Kluyveromyces marxianus* var. *lactis** (d-calf prochymosin gene)	
Chymotrypsin	Protease	Bovine or porcine pancreatic extract	None
Ficin	Protease	Figs: *Ficus* sp.	None

α-Galactosidase	Carbohydrase	(1) *Mortierella vinacea* var. *raffinoseutilizer*	α-D-Galactoside galactohydrolase
		(2) *Aspergillus*	

TRIVIAL NAME	CLASSIFICATION	SOURCE	NAMES (IUB)[a]
β-Glucanase	Carbohydrase	(1) *Aspergillus niger* var.	1,3-(1,3;1,4)-β-D-Glucan 3(4)-glucanohydrolase
		(2) *Bacillus subtilis* var.	
		(3) *Trichoderma longibrachiatum*	
Glucoamylase (Amyloglucosidase)	Carbohydrase	(1) *Aspergillus niger* var.	1,4-α-D-Glucan glucohydrolase
		(2) *Aspergillus oryzae* var.	
		(3) *Rhizopus oryzae* var.	
		(4) *Rhizopus niveus*	
Glucose Isomerase	Isomerase	(1) *Actinoplanes missouriensis*	D-Xylose ketoisomerase
		(2) *Bacillus coagulans*	
		(3) *Streptomyces olivaceus*	
		(4) *Streptomyces olivochromogenus*	
		(5) *Streptomyces rubiginosus*	
		(6) *Streptomyces murinus*	
		(7) *Microbacterium arborescens*	

Glucose Oxidase	Oxidoreductase	*Aspergillus niger* var.	β-D-Glucose: oxygen1-oxidoreductase

TRIVIAL NAME	CLASSIFICATION	SOURCE	NAMES (IUB)[a]
β-D-Glucosidase	Carbohydrase	(1) *Aspergillus niger* var.	β-D-Glucoside glucohydrolase
		(2) *Trichoderma longibrachiatum*	
Hemicellulase	Carbohydrase	(1) *Aspergillus niger* var.	(1) α-L-Arabinofuranoside arabinofuranohydrolase
		(2) *Trichoderma longibrachiatum*	(2) 1,4-β-D-Mannan mannanohydrolase
			(3) 1,3-β-D-Xylan xylanohydrolase
			(4) 1,5-α-L-Arabinan arabinanohydrolase
Invertase	Carbohydrase	*Saccharomyces* sp. (*Kluyvero-myces*) and *Saccharomyces* sp (*cerevisiae*)	β-D-Fructofuranoside fructohydrolase
Lactase	Carbohydrase	(1) *Aspergillus niger* var.	β-D-Galactoside galactohydrolase
		(2) *Aspergillus oryzae* var.	
		(3) *Saccharomyces* sp.	
		(4) *Candida pseudotropicalis*	
		(5) *Kluyveromyces marxianus* var. *lactis*	

Lipase	Lipase	(1) Edible forestomach tissue of calves, kids, and lambs	(1) Carboxylic-ester hydrolase
		(2) Animal pancreatic tissues	(2) Triacylglycerol acylhydrolase

TRIVIAL NAME	CLASSIFICATION	SOURCE	NAMES (IUB)[a]
		(3) *Aspergillus oryzae* var.	
		(4) *Aspergillus niger* var.	
		(5) *Rhizomucor miehei*	
		(6) *Candida rugosa*	
Lysozyme	Lysozyme	Egg white	Peptidoglycan *N*-acetyl-muramoylhydrolase
Maltogenic Amylase	Carbohydrase	*Bacillus subtilis** (d-*Bacillus stearothermophilus*)	1,4-α-D-Glucan α-maltohydrolase
Pancreatin	Mixed carbohydrase, protease, and lipase	Bovine and porcine pancreatic tissue	(1) 1,4-α-D-Glucan glucanohydrolase
			(2) Triacylglycerol acylhydrolase
			(3) Protease
Papain	Protease	Papaya: *Carica papaya (L)*	None
Pectinase[b]	Carbohydrase	(1) *Aspergillus niger* var.	(1) Poly(1,4-α-D-galacturonide) glycanohydrolase
		(2) *Rhizopus oryzae* var.	(2) Pectin pectylhydrolase
			(3) Poly(1,4-α-D-glacturonide) lyase
			(4) Poly(methoxyl-L-

TRIVIAL NAME	CLASSIFICATION	SOURCE	galacturonide) lyase NAMES (IUB)[a]
Pepsin	Protease	Porcine or other animal stomach tissue	None
Phospholipase A_2	Lipase	Animal pancreatic tissue	Phosphatidylcholine 2-acylhydrolase
Phytase	Phosphatase	*Aspergillus niger* var.	(1) *myo*-Inositol-hexakisphosphate-3-phosphohydrolase
			(2) Orthophosphoric-mono ester phosphohydrolase
Protease (general)	Protease	(1) *Aspergillus niger* var.	None
		(2) *Aspergillus oryzae* var.	
		(3) *Bacillus subtilis* var.	
		(4) *Bacillus licheniformis* var.	
Pullulanase	Carbohydrase	*Bacillus acidopullulyticus*	α-Dextrin-6-glucanohydrolase
Rennet	Protease	(1) Fourth stomach of ruminant animals	None
		(2) *Endothia parasitica*	
		(3) *Rhizomucor miehei*	
		(4) *Rhizomucor pusillus* (Lindt)	
		(5) *Bacillus cereus*	

TRIVIAL NAME	CLASSIFICATION	SOURCE	NAMES (IUB)[a]
Trypsin	Protease	Animal pancreas	None

[a] *Enzyme Nomenclature: Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology,* Academic Press, New York, 1992.

* The asterisk indicates a genetically modified organism. The donor organism is listed after "d-". (d-*Bacillus brevis*).

[b] Usually a mixture of pectin depolymerase, pectin methylesterase, pectin lyase, and pectate lyase.

The following procedures are provided for application as necessary in determining compliance with the vendor's declared representations for enzyme activity. For all of the procedures use filtered, ultra-high purity water with a resistivity of 16–18 megohms. [NOTE—The enzyme assay procedures contained in Appendix V are not intended to be used solely with enzymes derived from any source organisms that may be listed in the individual assays, including those indicated by the title of the tests. Applicability of these enzyme assays is intended to be broad, and titles that designate an assay as "Fungal" or "Bacterial" are historical titles and not meant to be used to indicate that the test may be used only for fungally or bacterially derived enzymes, respectively. Where such designations are made in assay titles, or where individual organisms are listed in the assays themselves, such information should be considered informational and not a requirement.]

- **ACID PHOSPHATASE ACTIVITY**

Application and Principle: This procedure is used to determine acid phosphatase activity in preparations derived from *Aspergillus niger* var. The test is based on the enzymatic hydrolysis of *p*-nitrophenyl phosphate, followed by the measurement of the released inorganic phosphate.

Reagents and Solutions

Glycine buffer (0.2 M, pH 2.5): Dissolve 15.014 g of glycine (Merck, Catalog No. 4201) in about 800 mL of water. Adjust the pH to 2.5 with 1 M hydrochloric acid (consumption should be about 80 mL), and dilute to 1000 mL with water.

Substrate (30 mM): Dissolve 1.114 g of *p*-nitrophenyl phosphate (Boehringer, Catalog No. 738 352) in *Glycine buffer*, and adjust the volume to 100 mL with the buffer. Prepare a fresh substrate solution daily.

TCA solution: Dissolve 15 g of trichloroacetic acid in water, and dilute to 100 mL.

Ascorbic acid solution: Dissolve 10 g of ascorbic acid in water, and dilute to 100 mL. Store under refrigeration. The solution is stable for 7 days.

Ammonium molybdate solution: Dissolve 2.5 g of ammonium molybdate [$(NH_4)_6MoO_{24}{\cdot}4H_2O$] (Merck, Catalog No. 1182) in water, and dilute to 100 mL.

1 M Sulfuric acid: Stir 55.6 mL of concentrated sulfuric acid (H_2SO_4) (Merck, Catalog No. 731) into about 800 mL of water. Allow to cool, and make up to 1000 mL with water.

Reagent C: Mix 3 volumes of *1 M Sulfuric acid* with 1 volume of *Ammonium molybdate solution*, then add 1 volume of *Ascorbic acid solution*, and mix well. Prepare fresh daily.

Standard phosphate solution: Prepare a 9.0-mM phosphate stock solution. Dissolve and dilute 612.4 mg of potassium dihydrogen phosphate (KH_2PO_4) (dried in desiccator with silica) to 500 mL with water in a volumetric flask. Make the following dilutions in water from the stock solution, and use these as standards (see *Table 2*).

Table 2

Dilution	Phosphorus Concentration (nmol/mL)	Acid Phosphatase Activity (HFU/mL)
1:100	90	2400
1:200	45	1200
1:400	22.5	600

Pipet 4.0 mL of each dilution into two test tubes. Also pipet 4.0 mL of water into one tube (reagent blank). Add 4.0 mL of *Reagent C*, and mix. Incubate at 500 for 20 min, and cool to room temperature. Measure the absorbances at 820 nm against that of the reagent blank. Prepare a standard curve by plotting the absorbances against acid phosphatase activity [HFU (acid phosphatase unit)/mL]. Construct a new standard curve with each series of assays.

Test preparation: Prepare a solution of the enzyme preparation in the *Glycine buffer* so that 1 mL will contain between 600 and 2400 HFU/mL.

Procedure: Pipet 1.9 mL of *Substrate* in two test tubes. Add 2.0 mL of *TCA solution* to one of the tubes (blank), and mix. Put the tubes without *TCA Solution* in a water bath at 37° and let them equilibrate for 5 min. While using a stopwatch, start the hydrolysis by adding sequentially at proper intervals 0.1 mL of *Test preparation* to each tube, and mix. After exactly 15 min of incubation, stop the reaction by adding 2.0 mL of *TCA solution* to each tube. Mix, and cool to room temperature. Add 0.1 mL of *Test preparation* to the reagent blank tube (kept at room temperature), and mix. If precipitate occurs, separate it by centrifugation for 10 min at 2000 g. Pipet 0.4 mL of each sample after hydrolysis into separate test tubes. Add 3.6 mL of water to each tube. Add 4.0 mL of *Reagent C*, and mix. Incubate at 50° for 20 min, and cool to room temperature. Determine the absorbance against that of reagent blank at 820 nm.

Calculation: One acid phosphatase unit (HFU) is the amount of enzyme that liberates, under the conditions of the assay, inorganic phosphate from *p*-nitrophenyl phosphate at the rate of 1 nmol/min.

Subtract the blank absorbance from the sample absorbance (the difference should be between 0.100 and 1.000). Determine the acid phosphatase activity (HFU/mL) from the standard curve, and multiply by the dilution factor. For the activity of solid samples, use the following equation:

$$\text{HFU/g} = (\text{HFU/mL} \times F)/\text{g}$$

in which F is the dilution factor and g is the weight, in grams, of the sample.

- **Aminopeptidase (Leucine) Activity**

Application and Principle: This procedure is used to determine leucine aminopeptidase activity in enzyme preparations derived from *Lactococcus lactis*. The assay is based on the rate of absorbance change over 5 min at 30°; the change in absorbance is due to liberated *p*-nitroaniline from the hydrolysis of leucine *p*-nitroanilide.

Apparatus

Spectrophotometer: Use a spectrophotometer with temperature control, suitable for measuring absorbancies at 410 nm.

Cuvette: Use a 10-mm light path, quartz.

Thermometer: Use a partial immersion thermometer with a suitable range.

Vortex mixer: Use a standard, variable-speed mixer.

Reagents and Solutions

pH 7.0 phosphate buffer (100 mM): Dissolve 13.6 g of anhydrous potassium dihydrogen orthophosphate in water, and dilute to 1 L (*Solution A*). Dissolve 22.8 g of dipotassium hydrogen orthophosphate trihydrate in water, and dilute to 1 L (*Solution B*). Slowly add approximately 550 mL of *Solution B* to approximately 400 mL of *Solution A* until the pH of the buffer stabilizes at 7 ± 0.02.

Substrate solution: Dissolve 0.0200 g of leucine *p*-nitroanilide hydrochloride (Sigma Chemical Co., Catalog No. L2158) in 100 mL of *pH 7.0 Phosphate buffer*.

***p*-Nitroaniline stock solution:** Transfer 156.9 mg of *p*-nitroaniline (Aldrich Chemical Co., Catalog No. 18,531-0) to a 1-L volumetric flask, and dilute to volume with water. This solution is 1.1136 mM.

[**Caution—***p*-Nitroaniline is highly toxic. Avoid breathing its dust; avoid contact with skin, eyes, and clothing. Wash the affected area with water; for eyes seek medical attention.]

Standard *p*-nitroaniline solutions: Prepare the following dilutions of *p-Nitroaniline stock solution*: dilute 1 mL of p-*Nitroaniline stock solution* to 100 mL with *pH 7.0 Phosphate buffer* (*Solution 1*, 0.01136 mM); dilute 9 mL of *Solution 1* with 3 mL of *pH 7.0 Phosphate buffer* (*Solution 2*, 0.00852 mM); and dilute 5 mL of *Solution 1* with 5 mL of *pH 7.0 Phosphate buffer* (*Solution 3*, 0.00568 mM).

Sample solution: Prepare a solution in *pH 7.0 Phosphate buffer* that contains between 0.025 and 0.1 unit of aminopeptidase activity per mL.

Procedure

Determine the absorbance of each of the three standard *p*-nitroaniline dilutions (solutions *1*, *2*, and *3*) at 410 nm using *pH 7.0 Phosphate buffer* as the blank.

Pipet 3 mL of *Substrate solution* into a cuvette, insert a thermometer in each to ensure that the temperature of the solution is correct, and equilibrate in the spectrophotometer to 30° ± 0.2°. Add 150 µL of *Sample Solution* to the equilibrated *Substrate Solution*. Mix, and start recording the absorbance. Continue recording the absorbance for approximately 5 min; it should increase linearly with time. To determine the rate of change of absorbance, ignore the initial 0.5 min of the assay line, and use a period of at least 4 min to estimate the rate of change.

Calculation

One aminopeptidase activity unit (AP) is defined as the quantity of aminopeptidase required to liberate 1 µmol/min of leucine from leucine *p*-nitroanilide under the conditions of the assay at pH 7.0 and 30°.

For each of the diluted *Standard solutions—1*, *2*, and *3*—plot absorbance against *p*-nitroaniline mM concentration. The result is a straight line that passes through the origin. Calculate the millimolar extinction coefficient (ε) of each *Standard* p-*nitroaniline solution* using the following formula:

$$\varepsilon = A_N/C$$

in which A_N is the absorbance of the *Standard* p-*Nitroaniline Solution* at 410 nm and C is the millimolar concentration of *p*-nitroaniline of that solution. Average the three calculated values; this should result in a value of approximately 8.8. Calculate the activity of each sample taken by the equation:

$$\text{AP/g} = (\Delta A \times TCV \times 1000)/(\varepsilon \times SV \times C)$$

in which ΔA is the rate of change of absorbance per min; TCV is the total cuvette volume (3.150 mL); SV is the sample volume (0.150 mL); and C is the concentration, in milligrams per milliliter, of the sample.

- **α-Amylase Activity (Nonbacterial)**

Application and Principle: This procedure is used to determine the α-amylase activity of enzyme preparations derived from *Aspergillus niger* var.; *Aspergillus oryzae* var.; *Rhizopus oryzae*

var.; and barley malt. The assay is based on the time required to obtain a standard degree of hydrolysis of a starch solution at 30° ± 0.1°. The degree of hydrolysis is determined by comparing the iodine color of the hydrolysate with that of a standard.

Apparatus

Reference color standard: Use a special Alpha-Amylase Color Disk (Orbeco Analytical Systems, 185 Marine Street, Farmingdale, NY 11735, Catalog No. 620-S5). Alternatively, prepare a color standard by dissolving 25.0 g of cobaltous chloride ($CoCl_2 \cdot 6H_2O$) and 3.84 g of potassium dichromate in 100 mL of 0.01 N hydrochloric acid. This standard is stable indefinitely when stored in a stoppered bottle or comparator tube.

Comparator: Use either the standard Hellige comparator (Orbeco, Catalog No. 607) or the pocket comparator with prism attachment (Orbeco, Catalog No. 605AHT). The comparator should be illuminated with a 100-W frosted lamp placed 6 in. from the rear opal glass of the comparator and mounted so that direct rays from the lamp do not shine into the operator's eyes.

Comparator tubes: Use the precision-bored square tubes with a 13-mm viewing depth that are supplied with the Hellige comparator. Suitable tubes are also available from other apparatus suppliers (e.g., Thomas Scientific).

Reagents and Solutions

Buffer solution (pH 4.8): Dissolve 164 g of anhydrous sodium acetate in about 500 mL of water, add 120 mL of glacial acetic acid, and adjust the pH to 4.8 with glacial acetic acid. Dilute to 1000 mL with water, and mix.

β-Amylase solution: Dissolve into 5 mL of water a quantity of β-amylase, free from α-amylase activity (Sigma Chemical Co., Catalog No. A7005), equivalent to 250 mg of β-amylase with 2000° diastatic power.

Special starch: Use starch designated as "Starch (Lintner) Soluble" (Baker Analyzed Reagent, Catalog No. 4010). Before using new batches, test them in parallel with previous lots known to be satisfactory. Variations of more than ±3° diastatic power in the averages of a series of parallel tests indicate an unsuitable batch.

Buffered substrate solution: Disperse 10.0 g (dry-weight basis) of *Special starch* in 100 mL of cold water, and slowly pour the mixture into 300 mL of boiling water. Boil and stir for 1 to 2 min, then cool, and add 25 mL of *Buffer solution*, followed by all of the *β-Amylase solution*. Quantitatively transfer the mixture into a 500-mL volumetric flask with the aid of water saturated with toluene, dilute to volume with the same solvent, and mix. Store the solution at 30° ± 2° for NLT 18 h nor more than 72 h before use. (This solution is also known as "buffered limit dextrin substrate.")

Stock iodine solution: Dissolve 5.5 g of iodine and 11.0 g of potassium iodide in about 200 mL of water, dilute to 250 mL with water, and mix. Store in a dark bottle, and make a fresh solution every 30 days.

Dilute iodine solution: Dissolve 20 g of potassium iodide in 300 mL of water, and add 2.0 mL of *Stock iodine solution*. Quantitatively transfer the mixture into a 500-mL volumetric flask, dilute to volume with water, and mix. Prepare daily.

Sample preparation: Prepare a solution of the sample so that 5 mL of the final dilution will give an endpoint between 10 and 30 min under the conditions of the assay.

For barley malt, finely grind 25 g of the sample in a Miag-Seck mill (Buhler-Miag, Inc., P.O. Box 9497, Minneapolis, MN 55440). Quantitatively transfer the powder into a 1000-mL Erlenmeyer flask, add 500 mL of a 0.5% solution of sodium chloride, and allow the infusion to stand for 2.5 h at 30° ± 0.2°, agitating the contents by gently rotating the flask at 20-min intervals.

[**Caution—**Do not mix the infusion by inverting the flask. The quantity of the grist left adhering to the inner walls of the flask as a result of agitation must be as small as possible.]

Filter the infusion through a 32-cm fluted filter of Whatman No. 1, or equivalent, paper on a 20-cm funnel, returning the first 50 mL of filtrate to the filter. Collect the filtrate until 3 h have elapsed from the time the sodium chloride solution and the sample were first mixed. Pipet 20.0 mL of the filtered infusion into a 100-mL volumetric flask, dilute to volume with the 0.5% sodium chloride solution, and mix.

Procedure

Pipet 5.0 mL of *Dilute iodine solution* into a series of 13- × 100-mm test tubes, and place them in a water bath maintained at 30° ± 0.1°, allowing 20 tubes for each assay.

Pipet 20.0 mL of the *Buffered substrate solution*, previously heated in the water bath for 20 min, into a 50-mL Erlenmeyer flask, and add 5.0 mL of 0.5% sodium chloride solution, also previously heated in the water bath for 20 min. Place the flask in the water bath.

At zero time, rapidly pipet 5.0 mL of the *Sample preparation* into the equilibrated substrate, mix immediately by swirling, stopper the flask, and place it back in the water bath. After 10 min, transfer 1.0 mL of the reaction mixture from the 50-mL flask into one of the test tubes containing the *Dilute iodine solution*, shake the tube, then pour its contents into a *Comparator tube*, and immediately compare with the *Reference color standard* in the *Comparator*, using a tube of water behind the color disk.

[Note—Be certain that the pipet tip does not touch the iodine solution; carryback of iodine to the hydrolyzing mixture will interfere with enzyme action and will affect the results of the determination.]

In the same manner, repeat the transfer and comparison procedure at accurately timed intervals until the α-amylase color is reached, at which time record the elapsed time. In cases where two comparisons 30 s apart show that one is darker and the other lighter than the *Reference color standard*, record the endpoint to the nearest quarter min. Shake out the 13-mm *Comparator tube* between successive readings. Minimize slight differences in color discrimination between operators by using a prism attachment and by maintaining a 6- to 10-in. distance between the *Comparator* and the operator's eye.

Calculation

One α-amylase dextrinizing unit (DU) is defined as the quantity of α-amylase that will dextrinize soluble starch in the presence of an excess of β-amylase at the rate of 1 g/h at 30°.

Calculate the α-amylase dextrinizing units in the sample as follows:

$$\text{DU (solution)} = 24/(W \times T)$$

and

$$\text{DU (dry basis)} = \text{DU (solution)} \times 100/(100 - M)$$

in which W is the weight, in grams, of the enzyme sample added to the incubation mixture in the 5-mL aliquot of the *Sample preparation* used; T is the elapsed dextrinizing time, in min; 24 is the product of the weight of the starch substrate (0.4 g) and 60 min; and M is the percent moisture in the sample, determined by suitable means.

- **α-Amylase Activity (Bacterial)**

Application and Principle: This procedure is used to determine the α-amylase activity, expressed as bacterial amylase units (BAU), of enzyme preparations derived from *Bacillus subtilis* var., *Bacillus licheniformis* var., and *Bacillus stearothermophilus*. It is not applicable to products that contain β-amylase. The assay is based on the time required to obtain a standard degree of hydrolysis of a starch solution at 30° ± 0.1°. The degree of hydrolysis is determined by comparing the iodine color of the hydrolysate with that of a standard.

Apparatus

Use the *Reference Color Standard*, the *Comparator*, and the *Comparator Tubes* as described under *α-Amylase Activity (Nonbacterial)*, described in this Appendix, but use either daylight or daylight-type fluorescent lamps as the light source for the *Comparator*. (Incandescent lamps give slightly lower results.)

Reagents and Solutions

pH 6.6 buffer: Dissolve 9.1 g of potassium dihydrogen phosphate (KH_2PO_4) in sufficient water to make 1000 mL (*Solution A*). Dissolve 9.5 g of dibasic sodium phosphate (Na_2HPO_4) in sufficient water to make 1000 mL (*Solution B*). Add 400 mL of *Solution A* to 600 mL of *Solution B*, mix, and adjust the pH to 6.6, if necessary, by the addition of *Solution A* or *Solution B* as required.

Dilute iodine solution: Prepare as directed under *α-Amylase Activity (Nonbacterial)*.

Special starch: Use the material described under *α-Amylase Activity (Nonbacterial)*.

Starch substrate solution: Disperse 10.0 g (dry-weight basis) of *Special starch* in 100 mL of cold water, and slowly pour the mixture into 300 mL of boiling water. Boil and stir for 1 to 2 min, and then cool while continuously stirring. Quantitatively transfer the mixture into a 500-mL volumetric flask with the aid of water, add 10 mL of *pH 6.6 Buffer*, dilute to volume with water, and mix.

Sample preparation: Prepare a solution of the sample so that 10 mL of the final dilution will give an endpoint between 15 and 35 min under the conditions of the assay.

Procedure:

Pipet 5.0 mL of *Dilute iodine solution* into a series of 13- × 100-mm test tubes, and place them in a water bath maintained at 30° ± 0.1°, allowing 20 tubes for each assay.

Pipet 20.0 mL of the *Starch substrate solution* into a 50-mL Erlenmeyer flask, stopper, and allow to equilibrate for 20 min in the water bath at 30°.

At zero time, rapidly pipet 10.0 mL of the *Sample preparation* into the equilibrated mixture, and continue as directed in the *Procedure* under *α-Amylase Activity (Nonbacterial)*, beginning with ". . . mix immediately by swirling, stopper the flask. . . ."

Calculation:

One bacterial amylase unit (BAU) is defined as that quantity of enzyme that will dextrinize starch at the rate of 1 mg/min under the specified test conditions.

Calculate the α-amylase activity of the sample, expressed as BAU, by the formula:

$$BAU/g = 40F/T$$

in which 40 is a factor (400/10) derived from the 400 mg of starch (20 mL of a 2% solution) and the 10-mL aliquot of *Sample Preparation* used; F is the dilution factor (total dilution volume/sample weight, in grams); and T is the dextrinizing time, in min.

- **Catalase Activity**

Application and Principle: This procedure is used to determine the catalase activity, expressed as Baker Units, of preparations derived from *Aspergillus niger* var., bovine liver, or *Micrococcus lysodeikticus*. The assay is an exhaustion method based on the breakdown of hydrogen peroxide by catalase and the simultaneous breakdown of the catalase by the peroxide under controlled conditions.

Reagents and Solutions

Ammonium molybdate solution (1%): Dissolve 1.0 g of ammonium molybdate [$(NH_4)_6MoO_{24}\cdot 4H_2O$] (Merck, Catalog No. 1182) in water, and dilute to 100 mL.

0.250 N Sodium thiosulfate: Dissolve 62.5 g of sodium thiosulfate ($Na_2S_2O_3\cdot 5H_2O$) in 750 mL of recently boiled and cooled water, add 3.0 mL of 0.2 N sodium hydroxide as a stabilizer,

dilute to 1000 mL with water, and mix. Standardize as directed for *0.1N Sodium thiosulfate* (see *Solutions and Indicators*), and, if necessary, adjust to exactly 0.250 N.

Peroxide substrate solution: Dissolve 25.0 g of anhydrous dibasic sodium phosphate (Na_2HPO_4), or 70.8 g of $Na_2HPO_4 \cdot 12H_2O$, in about 1500 mL of water, and adjust to pH 7.0 ± 0.1 with 85% phosphoric acid. Cautiously add 100 mL of 30% hydrogen peroxide, dilute to 2000 mL in a graduate, and mix. Store in a clean amber bottle, loosely stoppered. The solution is stable for more than 1 week if kept at 5° in a full container. (With freshly prepared substrate, the blank will require about 16 mL of *0.250 N Sodium thiosulfate*. If the blank requires less than 14 mL, the substrate solution is unsuitable and should be prepared fresh again. The sample titration must be between 50% and 80% of that required for the blank.)

Procedure

Pipet an aliquot of NMT 1.0 mL of the sample, previously diluted to contain approximately 3.5 Baker Units of catalase, into a 200-mL beaker. Rapidly add 100 mL of *Peroxide substrate solution*, previously adjusted to 25°, and stir immediately for 5 to 10 s. Cover the beaker, and incubate at 25° ± 1° until the reaction is completed. Stir vigorously for 5 s, and then pipet 4.0 mL from the beaker into a 50-mL Erlenmeyer flask. Add 5 mL of 2 N sulfuric acid to the flask, mix, then add 5.0 mL of 40% potassium iodide solution, freshly prepared, and 1 drop of *Ammonium molybdate solution* (1%), and mix. While continuing to mix, titrate rapidly to a colorless endpoint with *0.250 N Sodium thiosulfate*, recording the volume, in milliliters, required as *S*. Perform a blank determination with 4.0 mL of *Peroxide substrate solution*, and record the volume required, in milliliters, as *B*.

[Note—When preparations derived from beef liver are tested, the reaction is complete within 30 min. Preparations derived from *Aspergillus* and other sources may require up to 1 h. In assaying an enzyme of unknown origin, run a titration after 30 min and then at 10-min intervals thereafter. The reaction is complete when two consecutive titrations are the same.]

Calculation

One Baker Unit is defined as the amount of catalase that will decompose 264 mg of hydrogen peroxide under the conditions of the assay.

Calculate the activity of the sample by the equation:

$$\text{Baker Units/g or mL} = 0.4(B - S) \times (1/C)$$

in which *C* is the milliliters of aliquot of original enzyme preparation added to each 100 mL of *Peroxide Substrate Solution*, or when 1 mL of diluted enzyme is used, *C* is the dilution factor; *B* is the volume, in milliliters, as defined above; and *S* is the milliliters of *0.250 N Sodium thiosulfate*, as defined above.

- **Cellulase Activity**

Application and Principle: This assay is based on the enzymatic hydrolysis of the interior β-1,4-glucosidic bonds of a defined carboxymethyl cellulose substrate at pH 4.5 and at 40°. The corresponding reduction in substrate viscosity is determined with a calibrated viscometer.

Apparatus

Calibrated viscometer: Use a size 100 Calibrated Cannon-Fenske Type Viscometer, or its equivalent (Scientific Products, Catalog No. P2885-100).

Constant-Temperature glass water bath (40° ± 0.1°): Use a constant-temperature glass water bath, or its equivalent (Scientific Products, Catalog No. W3520-10).

Stopwatches: Use two stopwatches, *Stopwatch No. 1*, calibrated in $^1/_{10}$ min for determining the reaction time (T_r), and *Stopwatch No. 2*, calibrated in ⅕ s for determining the efflux time (T_t).

Waring blender: Use a two-speed Waring blender, or its equivalent (Scientific Products, Catalog No. 58350-1).

Reagents and Solutions

Acetic acid solution (2 N): While agitating a 1-L beaker containing 800 mL of water, carefully add 116 mL of glacial acetic acid. Cool to room temperature. Quantitatively transfer the solution to a 1-L volumetric flask, and dilute to volume with water.

Sodium acetate solution (2 N): Dissolve 272.16 g of sodium acetate trihydrate in approximately 800 mL of water contained in a 1-L beaker. Quantitatively transfer to a 1-L volumetric flask, and dilute to volume with water.

Acetic acid solution (0.4 N): Transfer 200 mL of *Acetic acid solution (2 N)* into a 1-L volumetric flask, and dilute to volume with water.

Sodium acetate solution (0.4 N): Transfer 200 mL of *Sodium acetate solution (2 N)* into a 1-L volumetric flask, and dilute to volume with water.

Acetate buffer (pH 4.5): Using a standardized pH meter, add *Sodium acetate dolution (0.4 N)* with continuous agitation to 400 mL of *Acetic acid solution (0.4 N)* in a suitable flask until the pH is 4.5 ± 0.05.

Sodium carboxymethylcellulose: Use sodium carboxymethylcellulose (Hercules, Inc., CMC Type 7HF).

Sodium carboxymethylcellulose substrate (0.2% w/v): Transfer 200 mL of water into the bowl of the Waring blender. With the blender on low speed, slowly disperse 1.0 g (moisture-free basis) of the *Sodium carboxymethylcellulose* into the bowl, being careful not to splash out any of the liquid. Using a rubber policeman, wash down the sides of the glass bowl with water. Place the top on the bowl and blend at high speed for 1 min. Quantitatively transfer to a 500-mL volumetric flask, and dilute to volume with water. Filter the substrate through gauze before use.

Sample preparation: Prepare an enzyme solution so that 1 mL of the final dilution will produce a relative fluidity change between 0.18 and 0.22 in 5 min under the conditions of the assay. Weigh the enzyme, and quantitatively transfer it to a glass mortar. Triturate with water and quantitatively transfer the mixture to an appropriate volumetric flask. Dilute to volume with water, and filter the enzyme solution through Whatman No. 1 filter paper before use.

Procedure

Place the *Calibrated viscometer* in the 40° ± 0.1° water bath in an exactly vertical position. Use only a scrupulously clean viscometer. (To clean the viscometer, draw a large volume of detergent solution followed by water through the viscometer by using an aspirator with a rubber tube connected to the narrow arm of the viscometer.)

Pipet 20 mL of filtered *Sodium carboxymethylcellulose substrate* and 4 mL of *Acetate buffer* into a 50-mL Erlenmeyer flask. Allow at least two flasks for each enzyme sample and one flask for a substrate blank. Stopper the flasks, and equilibrate them in the water bath for 15 min.

At zero time, pipet 1 mL of the enzyme solution into the equilibrated substrate. Start stopwatch no. 1, and mix the solution thoroughly. Immediately pipet 10 mL of the reaction mixture into the wide arm of the viscometer.

After approximately 2 min, apply suction with a rubber tube connected to the narrow arm of the viscometer, drawing the reaction mixture above the upper mark into the driving fluid head. Measure the efflux time by allowing the reaction mixture to freely flow down past the upper mark. As the meniscus of the reaction mixture falls past the upper mark, start stopwatch no. 2. At the same time, record the reaction time, in min, from stopwatch no. 1 (T_r). As the meniscus of the reaction mixture falls past the lower mark, record the time, in seconds, from stopwatch no. 2 (T_t). Repeat the final step until a total of four determinations is obtained over a reaction time (T_r) of NMT 15 min.

Prepare a substrate blank by pipetting 1 mL of water into 24 mL of buffered substrate. Pipet 10 mL of the reaction mixture into the wide arm of the viscometer. Determine the time (T_s) in seconds required for the meniscus to fall between the two marks. Use an average of five determinations for (T_s).

Prepare a water blank by pipetting 10 mL of equilibrated water into the wide arm of the viscometer. Determine the time (T_w) in seconds required for the meniscus to fall between the two marks. Use an average of five determinations for (T_w).

Calculations

One Cellulase Unit (CU) is defined as the amount of activity that will produce a relative fluidity change of 1 in 5 min in a defined carboxymethyl cellulose substrate under the conditions of the assay.

Calculate the relative fluidities (F_r) and the (T_n) values for each of the four efflux times (T_t) and reaction times (T_r) as follows:

$$F_r = (T_s - T_w)/(T_t - T_w)$$

$$T_n = ½(T_t/60 \text{ s/min}) + T_r = (T_t/120) + T_r$$

in which F_r is the relative fluidity for each reaction time; T_s is the average efflux time, in seconds, for the substrate blank; T_w is the average efflux time, in seconds, for the water blank; T_t is the efflux time, in seconds, of reaction mixture; T_r is the elapsed time, in min, from zero time, that is, the time from addition of the enzyme solution to the buffered substrate until the beginning of the measurement of efflux time (T_t); and T_n is the reaction time, in min (T_r), plus one-half of the efflux time (T_t), converted to min.

Plot the four relative fluidities (F_r) as the ordinate against the four reaction times (T_n) as the abscissa. A straight line should be obtained. The slope of this line corresponds to the relative fluidity change per min and is proportional to the enzyme concentration. The slope of the best line through a series of experimental points is a better criterion of enzyme activity than is a single relative fluidity value. From the graph, determine the F_r values at 10 and 5 min. They should have a difference in fluidity of NMT 0.22 or NLT 0.18. Calculate the activity of the enzyme unknown as follows:

$$\text{CU/g} = [1000(F_{r10} - F_{r5})]/W$$

in which F_{r5} is the relative fluidity at 5 min of reaction time; F_{r10} is the relative fluidity at 10 min of reaction time; 1000 is the milligrams per gram; and W is the weight, in milligrams, of enzyme added to the reaction mixture in a 1-mL aliquot of enzyme solution.

- **Chymotrypsin Activity**

Application and Principle: This procedure is used to determine chymotrypsin activity in chymotrypsin preparations derived from purified extracts of porcine or bovine pancreas.

Reagents and Solutions

0.15 M Phosphate buffer (pH 7.0): Dissolve 4.54 g of monobasic potassium phosphate in water, and dilute to 500 mL. Dissolve 4.73 g of anhydrous dibasic sodium phosphate in water, and dilute to 500 mL. Mix 38.0 mL of the monobasic potassium phosphate solution with 61.1 mL of the dibasic sodium phosphate solution. Adjust the pH of the mixture to 7.0 by the dropwise addition of the dibasic sodium phosphate solution, if necessary.

Substrate solution: Dissolve 23.7 mg of *N*-acetyl-*L*-tyrosine ethyl ester in about 50 mL of the *0.15 M Phosphate buffer* with warming. When the solution has cooled, dilute to 100.0 mL with the *0.15 M Phosphate buffer*.

Sample preparation: Dissolve a sufficient amount of sample, accurately weighed, in 0.001 N hydrochloric acid to produce a solution containing between 12 and 16 USP Chymotrypsin Units per milliliter. This solution should cause a change in absorbance between 0.008 and 0.012 in a 30-s interval.

Procedure

Conduct the assay in a suitable spectrophotometer equipped to maintain a temperature of 24° ± 0.1° in the cell compartment. Determine the temperature before and after measuring the absorbance to ensure that the temperature does not change more than 0.5° during the assay. Pipet 0.2 mL of the 0.001 N hydrochloric acid and 3.0 mL of the *Substrate solution* into a 1-cm cell. Place the cell in the spectrophotometer, and adjust the instrument so that the absorbance will read 0.200 at 237 nm. Pipet 0.2 mL of the *Sample preparation* into a second cell, add 3.0 mL of the *Substrate solution*, and place the cell in the spectrophotometer. Begin timing the reaction from the addition of the *Substrate solution*. Read the absorbance at 30-s intervals for at least 5 min. Repeat the procedure at least once. If the rate of change fails to remain constant for at least 3 min, repeat the test, and if necessary, use a lower sample concentration. The duplicate determinations at the same sample concentration should match the first determination in rate of absorbance change.

Calculations

One USP Chymotrypsin Unit is defined as the activity causing a change in absorbance at the rate 0.0075/min under the conditions of the assay. Determine the average absorbance change per min using only those values within the 3-min portion of the curve where the rate of change is constant. Plot a curve of absorbance against time.

Calculate the number of Chymotrypsin Units per milligram by the formula:

$$\text{Result} = (A_2 - A_1)/(0.0075TW)$$

in which A_2 is the straight-line initial absorbance reading; A_1 is the straight-line final absorbance reading; T is the elapsed time, in min; and W is the weight, in milligrams, of the sample in the volume of solution used to determine the absorbance.

- **Diastase Activity (Diastatic Power)**

Application and Principle: This procedure is used to determine the amylase activity of barley malt and other enzyme preparations. The assay is based on a 30-min hydrolysis of a starch substrate at pH 4.6 and 20°. The reducing sugar groups produced on hydrolysis are measured in a titrimetric procedure using alkaline ferricyanide.

Apparatus

Mill: Use a laboratory mill of the type Miag-Seck, for fine grinding of malt (Buhler Miag, Inc.).

Reagents and Solutions

Acetate buffer solution:

Dissolve 68 g of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) in 500 mL of 1 N acetic acid in a 1000-mL volumetric flask, dilute to volume with water, and mix.

Special starch: Use the material described under *α-Amylase activity (nonbacterial)*.

Starch substrate solution: Disperse 20.0 g (dry-weight basis) of *Special starch* in 50 mL of water, mix to a fine paste, and pour slowly into 750 mL of boiling water. Boil and stir for 2 min, cool, add 20 mL of *Acetate buffer solution*, and mix. Quantitatively transfer into a 1000-mL volumetric flask, dilute to volume with water, and mix.

Acetic acid–potassium chloride–zinc sulfate solution (A-P-Z): Dissolve 70 g of potassium chloride and 20 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) in 700 mL of water in a 1000-mL volumetric flask, add 200 mL of glacial acetic acid, dilute to volume with water, and mix.

Alkaline ferricyanide solution (0.05 N): Dissolve 16.5 g of potassium ferricyanide [$K_3Fe(CN)_6$] and 22 g of anhydrous sodium carbonate in 800 mL of water in a 1000-mL volumetric flask, dilute to volume with water, and mix.

Potassium iodide solution: Dissolve 50 g of potassium iodide in 50 mL of water in a 100-mL volumetric flask, dilute to volume with water, and mix. Add 2 drops of 50% sodium hydroxide solution, and mix. The solution should be colorless.

Sample Preparation

Malt samples: Grind 30 g of the sample to a fine powder in a Maig-Seck mill. Accurately weigh 25 g of the powder, and transfer it into a 1000-mL Erlenmeyer flask. Add 500 mL of a 0.5% sodium chloride solution, and allow the infusion to stand for 2.5 h at 20° ± 0.2°, agitating the contents by gently rotating the flask at 20-min intervals.

[NOTE—Do not mix the infusion by inverting the flask. The quantity of grist left adhering to the inner walls of the flask as a result of agitation must be as small as possible. Gently swirl the contents of the flask without splashing them against the walls to mix sufficiently.]

Filter the infusion through a 32-cm fluted filter of Whatman No. 1, or equivalent, paper on a 20-cm funnel, returning the first 50 mL of filtrate to the filter. Place a watch glass over the funnel, and use a suitable cover around the stem and over the receiver to reduce evaporation losses during filtration. Collect the filtrate until 30 min of filtration time have elapsed. Pipet 20.0 mL of the filtrate into a 100-mL volumetric flask, dilute to volume with 0.5% sodium chloride solution, and mix.

Other enzyme preparations: Prepare a solution so that 10 mL of the final dilution will give a diastatic power (DP) value between 2° and 150°.

Procedure

Pipet 10.0 mL of the *Sample preparation* into a 250-mL volumetric flask, and at zero time, add 200 mL of *Starch substrate solution*, previously equilibrated for 30 min in a water bath maintained at 20° ± 0.2°. Start the stopwatch at zero time.

Place the mixture in the water bath at 20°, and allow it to cool for exactly 30 min, then add 20.0 mL of 0.5 N sodium hydroxide, dilute to volume with water, and mix.

Prepare a blank by adding 20.0 mL of 0.5 N sodium hydroxide to a 250-mL volumetric flask, followed by 10.0 mL of the *Sample preparation*. Swirl to mix, add 200 mL of *Starch substrate solution*, dilute to volume with water, and mix.

Pipet 5.0 mL of the sample digestion mixture into a 125-mL Erlenmeyer flask, add 10.0 mL of *Alkaline ferricyanide solution*, and swirl to mix. Heat the flask for exactly 20 min in a boiling water bath, and then cool to room temperature. Add 25 mL of *A-P-Z solution*, followed by 1 mL of *Potassium iodide solution*, and swirl to mix. Titrate with 0.05 N sodium thiosulfate to the complete disappearance of the blue color, recording the volume, in milliliters, of 0.05 N sodium thiosulfate required as S.

Treat the blank solution in the same manner as described for the sample, recording the volume, in milliliters, of 0.05 N sodium thiosulfate required as B.

Calculation

One unit of diastase activity, expressed as degrees diastatic power (DP°), is defined as that amount of enzyme contained in 0.1 mL of a 5% solution of the sample enzyme preparation that will produce sufficient reducing sugars to reduce 5 mL of Fehling's solution when the sample is incubated with 100 mL of the substrate for 1 h at 20°.

[NOTE—The definition of the unit does not correspond to the method of the determination.]

Calculate the diastase activity, expressed as DP°, of the sample by the formulas:

$$\text{DP°, as-is basis} = (B - S) \times 23$$

and

$$\text{DP°, dry basis} = \text{DP°, as-is basis} \times 100/(100 - M)$$

in which 23 is a factor, determined by collaborative study, required to convert to the units of the definition, and M is the percent moisture of the sample, determined by suitable means.

- **α-Galactosidase Activity**

Application and Principle: Use this procedure to determine α-galactosidase activity in enzyme preparations derived from *Aspergillus niger* var. The assay is based on a 15-min hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside followed by spectrophotometric measurement of the liberated *p*-nitrophenol.

Reagents and Solutions

Acetate buffer: Dissolve 11.55 mL of glacial acetic acid in water, and dilute to 1 L (*Solution A*). Dissolve 16.4 g of sodium acetate in water, and dilute to 1 L (*Solution B*). Mix 7.5 mL of *Solution A* and 42.5 mL of *Solution B*, and dilute to 200 mL with water. Adjust the pH of this solution to 5.5 with either *Solution A* or *Solution B* as necessary.

Substrate solution: Dissolve 0.210 g of *p*-nitrophenyl-α-D-galactopyranoside (Sigma Chemical Co., Catalog No. 877, or equivalent) in and dilute to 100 mL with *Acetate buffer*.

Borax buffer: Dissolve 47.63 g of sodium borate decahydrate in warm water. Cool to room temperature. Add 20 mL of 4 N sodium hydroxide solution, adjust the pH of the solution to 9.7 with 4 N sodium hydroxide, and dilute to 2 L with water.

***p*-Nitrophenol stock solution:** Dissolve 0.0334 g of *p*-nitrophenol (Aldrich Chemical Co., Catalog No. 24,132-6, or equivalent) in and dilute to 1 L with water. This solution contains 0.24 µmol of *p*-nitrophenol per milliliter of water.

Preparation of Standards and Samples

Standards: Prepare the following dilutions of p-*Nitrophenol stock solution* with water: 100:50 (v/v) (0.16 µmol/mL); 50:100 (v/v) (0.08 µmol/mL); and 25:125 (v/v) (0.04 µmol/mL). Transfer 2.0 mL of the *Substrate solution* to each of five separate test tubes. Add 1 mL of the p-*Nitrophenol stock solution* to the first tube, 1.0 mL of each dilution to the next three tubes, and 1.0 mL of water to the fifth tube. Add 5.0 mL of *Borax buffer* to each tube, and mix.

Samples: Prepare a solution of α-galactosidase sample in *Acetate buffer* that contains between 0.008 and 0.024 galactosidase units of activity per milliliter.

Procedure

Equilibrate the *Substrate solution* in a water bath at 37° ± 0.2° for at least 15 min. For active samples, transfer 1.0 mL of each sample to separate test tubes and equilibrate in the 37° ± 0.2° water bath. At zero time, add 2.0 mL of *Substrate solution*, mix, and return to the water bath. After exactly 15.0 min, add 5.0 mL of *Borax buffer* to each tube, mix, and remove from the water bath.

For sample blanks, transfer, in sequence, 1.0 mL of each sample to separate test tubes, add 5.0 mL of *Borax Buffer*, and mix. Add 2.0 mL of *Substrate solution* to each tube, and mix.

Measure the absorbance of each standard sample and blank at 405 nm versus that of water. Determine the absorbances of all solutions within 30 min of completing the tests.

Calculations

One galactosidase activity unit (GalU) is defined as the quantity of the enzyme that will liberate *p*-nitrophenol at the rate of 1 µmol/min under the conditions of the assay.

Calculate the factor ε for the *p*-nitrophenol standards using the following equation:

$$\text{Result} = \varepsilon = A_N/C$$

in which A_N is the absorbance of the *p*-nitrophenol standards at 405 nm, and C is the concentration, in millimoles per milliliter, of *p*-nitrophenol.

Because the averaged millimolar extinction coefficient of *p*-nitrophenol at 405 nm is 18.3, ε should be approximately 2.29 [or (18.3)/8].

$$\text{GalU/g} = [(A_S - A_B) \times F]/(\varepsilon \times T \times M),$$

in which A_S is the sample absorbance; A_B is the blank absorbance; F is the appropriate dilution factor; T is the reaction time, in min; M is the weight, in grams, of the sample; and ε is a factor calculated above for the *p*-nitrophenol standards (proportional to the millimolar extinction coefficient for *p*-nitrophenol).

- **β-Glucanase Activity**

Application and Principle: This procedure is used to determine β-glucanase activity of enzyme preparations derived from *Aspergillus niger* var. and *Bacillus subtilis* var. The assay is based on a 15-min hydrolysis of lichenin substrate at 40° and at pH 6.5. The increase in reducing power due to liberated reducing groups is measured by the neocuproine method.

Reagents and Solutions

Phosphate buffer: Dissolve 13.6 g of monobasic potassium phosphate in about 1900 mL of water, add 70% sodium hydroxide solution until the pH is 6.5 ± 0.05, then transfer the solution into a 2000-mL volumetric flask, dilute to volume with water, and mix.

Neocuproine solution A: Dissolve 40.0 g of anhydrous sodium carbonate, 16.0 g of glycine, and 450 mg of cupric sulfate pentahydrate in about 600 mL of water. Transfer the solution into a 1000-mL volumetric flask, dilute to volume with water, and mix.

Neocuproine solution B: Dissolve 600 mg of neocuproine hydrochloride in about 400 mL of water, transfer the solution into a 500-mL volumetric flask, dilute to volume with water, and mix. Discard when a yellow color develops.

Lichenin substrate: Grind 150 mg of lichenin (Sigma Chemical Co., Catalog No. L-6133, or equivalent) to a fine powder in a mortar, and dissolve it in about 50 mL of water at about 85°. After solution is complete (20 to 30 min), add 90 mg of sodium borohydride and continue heating below the boiling point for 1 h. Add 15 g of Amberlite MB-3, or an equivalent ion-exchange resin, and stir continuously for 30 min. Filter with the aid of a vacuum through Whatman No. 1 filter paper, or equivalent, in a Büchner funnel, and wash the paper with about 20 mL of water. Add 680 mg of monobasic potassium phosphate to the filtrate, and refilter through a 0.22-µm Millipore filter pad, or equivalent. Wash the pad with 10 mL of water, and adjust the pH of the filtrate to 6.5 ± 0.05 with 1 N sodium hydroxide or 1 N hydrochloric acid. Transfer the filtrate into a 100-mL volumetric flask, dilute to volume with water, and mix. Store at 2° to 4° for NMT 3 days.

Glucose standard solution: Dissolve 36.0 mg of anhydrous dextrose in *Phosphate buffer* in a 1000-mL volumetric flask, dilute to volume with water, and mix.

Test preparation: Prepare a solution from the enzyme preparation sample so that 1 mL of the final dilution will contain between 0.01 and 0.02 β-glucanase units. Weigh the sample, transfer it into a volumetric flask of appropriate size, dilute to volume with *Phosphate buffer*, and mix.

Procedure

Pipet 2 mL of *Lichenin substrate* into each of four separate test tubes graduated at 25 mL, and heat the tubes in a water bath at 40° for 10 to 15 min to equilibrate.

After equilibration, add 1 mL of *Phosphate buffer* to tube 1 (substrate blank), 1 mL of *Glucose standard solution* to tube 2 (glucose standard), 4 mL of *Neocuproine sSolution A* and 1 mL of the *Test preparation* to tube 3 (enzyme blank), and 1 mL of the *Test preparation* to tube 4 (sample). Prepare a fifth tube for the buffer blank, and add 3 mL of *Phosphate buffer*.

Incubate the five tubes at 40° for exactly 15 min, and then add 4 mL of *Neocuproine solution A* to tubes 1, 2, 4, and 5. Add 4 mL of *Neocuproine solution B* to all five tubes, and cap each with a

suitably sized glass marble.
[**Caution—**Do not use rubber stoppers.]

Heat the tubes in a vigorously boiling water bath for exactly 12 min to develop color, then cool to room temperature in cold water, and adjust the volume of each to 25 mL with water. Cap the tubes with Parafilm, or other suitable closure, and mix by inverting several times.
Determine the absorbance of each solution at 450 nm in 1-cm cells, with a suitable spectrophotometer, against the buffer blank in tube 5.

Calculation

One β-glucanase unit (BGU) is defined as that quantity of enzyme that will liberate reducing sugar (as glucose equivalence) at a rate of 1 µmol/min under the conditions of the assay.
Calculate the activity of the enzyme preparation taken for analysis as follows:

$$\text{BGU} = [(A_4 - A_3) \times 36 \times 10^6]/[(A_2 - A_1) \times 180 \times 15 \times \mu\text{g sample}]$$

in which A_4 is the absorbance of the sample (tube 4), A_3 is the absorbance of the enzyme blank (tube 3), A_2 is the absorbance of the glucose standard (tube 2), A_1 is the absorbance of the substrate blank (tube 1), 36 is the micrograms of glucose in the *Glucose standard solution*, 10^6 is the factor converting micrograms to grams, 180 is the weight of 1 µmol of glucose, and 15 is the reaction time, in min.

- **Glucomylase Activity (Amyloglucosidase Activity)**

Application and Principle: This procedure is used to determine the glucoamylase activity of preparations derived from *Aspergillus niger* var., but it may be modified to determine preparations derived from *Aspergillus oryzae* var. and *Rhizopus oryzae* var. (as indicated by the variations in the text below). The sample hydrolyzes *p*-nitrophenyl-α-D-glucopyranoside (PNPG) to *p*-nitrophenol (PNP) and glucose at pH 4.3 and 50°.
Use the quantity of PNP liberated per unit of time to calculate the enzyme activity. Measure the PNP liberated against a quantity of a standard preparation of PNP by measuring the absorbance of the solutions at 400 nm after adjusting the pH of the reaction mixture to pH 8.0.

[Note—Use a pH of 5.0 when testing preparations derived from *Aspergillus oryzae* var. or *Rhizopus oryzae* var.]

Apparatus

Water bath: Use an open, circulating water bath with control accuracy of at least ±0.1°.

Spectrophotometer: Use a spectrophotometer suitable for measuring absorbances at 400 nm.

Cuvettes: Use 10-mm light-path fused quartz.

Thermometer: Use a partial immersion thermometer with a suitable range, graduated in 1/10°.

Timer: Use a solid-state timer, model 69240 (GCS Corporation, Precision Scientific Group), or equivalent, accurate to ±0.01 min in 240 min.

Vortex mixer: Use a standard variable-speed mixer.

Reagents and Solutions

***p*-Nitrophenol stock solution (PNP) (0.001 M):** Dissolve 139.11 mg of *p*-nitrophenol previously dried (60°, maximum 4 h) into water, and dilute to 1000 mL.

[**Caution—**Avoid contact with skin. If contact occurs, wash the affected area with water. Work in a well-ventilated area.]

Acetate buffer solution (0.1 M): Dissolve 4.4 g of sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) in approximately 800 mL of water, and add 4.5 mL of acetic acid ($C_2H_4O_2$). Adjust to a

pH of 4.5 ± 0.05 by adding either sodium acetate or glacial acetic acid as required. Dilute to 1 L.

[NOTE—Use a pH of 5.0 when testing preparations derived from *Aspergillus oryzae* var. or *Rhizopus oryzae* var.]

The pH optimum is 5.0 for *Aspergillus oryzae* var.—or *Rhizopus oryzae* var.—derived preparations.

Sodium carbonate solution (0.3 M): Dissolve 15.9 g of sodium carbonate (Na_2CO_3) in water, and dilute to 500 mL.

***p*-Nitrophenyl-α-D-glucopyranoside solution (PNPG):** Dissolve 100.0 mg of PNPG (Sigma Chemical Co., Catalog No. N1377) in acetate buffer, and dilute to 100 mL.

Preparation of Standards and Samples

Standards: Dilute three portions of *PNP stock solution* to produce standards for the standard curve. Add 3 mL of the *PNP stock solution* to 125 mL of *Sodium carbonate solution*, and dilute with water to 100 mL to produce the second standard, containing 0.02 µmol/mL. Add 5 mL of *PNP stock solutions* to 25 mL of *Sodium carbonate solution*, and dilute with water to 100 mL to produce the third standard, containing 0.05 µmol/mL.

Sample solution: Dilute 1.00 ± 0.01 g of sample in sufficient *Acetate buffer solution* to produce a solution that contains between 0.05 and 0.15 glucoamylase units of activity per mL.

Procedure

Measure absorbances of each of the three *PNP standard solutions* to calculate the molar extinction coefficient. Equilibrate the *PNPG solution* in a 50° water bath for at least 15 min. For active samples, transfer 2.0 mL of the *Sample solution* to a test tube. Loosely stopper, and place the tube in the water bath to equilibrate for 5 min. At zero time, add 2.0 mL of *PNPG solution*, and mix at moderate speed on a vortex mixer. Return the mixture to the water bath. Exactly 10.0 min later, add 3.0 mL of the *Sodium carbonate solution*, mix on the vortex, and remove from the water bath.

For sample blanks, transfer 2.0 mL of the *Sample solution* and 3.0 mL of the *Sodium carbonate solution* into a test tube, and mix. Add 2.0 mL of *PNPG solution*, and mix. Measure the absorbance of each sample and the blank versus water in a 10-mm cell.

[NOTE—Determine the absorbance of the sample and blank solutions NMT 20 min after adding *Sodium carbonate solution*.]

Calculations

One unit of glucoamylase activity is defined as the amount of glucoamylase that will liberate 0.1 µmol/min of *p*-nitrophenol from the *PNPG solution* under the conditions of the assay.

Calculate the millimolar extinction of the PNP standards:

$$\varepsilon = A_n/C$$

in which A_n is the absorbance of the *p*-nitrophenol standard, at 400 nm; and C is the concentration, in µmol/mL, of *p*-nitrophenol.

The averaged millimolar extinction coefficient, M, should be approximately 18.2.

$$\text{Glucoamylase U/g} = [(A_S - A_B) \times 7 \times F]/M \times 10 \times 0.10 \times W \times 2,$$

in which A_S is the sample absorbance; A_B is the blank absorbance; F is the appropriate dilution factor; W is the weight of sample, in g; 7 is the final volume of the test solutions; 10 is the reaction time, in min; 0.10 is the amount of PNP liberated, in µmol/min/unit of enzyme; 2 is the sample aliquot, in mL; and M is the millimolar extinction coefficient.

- **GLUCOSE ISOMERASE ACTIVITY**

[NOTE—Glucose isomerase activity of the commercial enzyme is usually determined on the enzyme that has been immobilized by binding with a polymer matrix or other suitable material. The following method is designed for use with such preparations.]

Application and Principle: Use this procedure to determine glucose isomerase preparations derived from *Actinoplanes missouriensis*, *Bacillus coagulans*, *Microbacterium arborescens*, *Streptomyces murinus*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, and *Streptomyces rubiginosus*. It is based on measurement of the rate of conversion of glucose to fructose in a packed-bed reactor. The procedure as outlined approximates an initial velocity assay method. Specific conditions are glucose concentration, 45% w/w; pH (inlet), measured at room temperature in the 7.0 to 8.5 range, as specified; temperature, 60.0°; and magnesium concentration, 4×10^{-3} M.

The optimum conditions for enzymes from different microbial sources and methods of preparation may vary; therefore, if the manufacturer recommends different pH conditions, buffering systems, or methods of sample preparation, use such variations in the instructions given in the text.

Apparatus

Column Assembly and Apparatus:

[NOTE—Make all connections with inert tubing, glass, or plastic as appropriate.]

The column assembly is shown in *Figure 1*. Use a 2.5- × 40-cm glass column provided with a coarse, sintered-glass bottom and a water jacket connected to a constant-temperature water bath, maintained at 60.0°, by means of a circulating pump. Connect the top of the column to a variable-speed peristaltic pump having a maximum flow rate of 800 mL/h. The diameter of the tubing with which the peristaltic pump is fitted should permit variation of the pumping volume from 60 to 150 mL/h. Connect the outlet of the column with a collecting vessel.

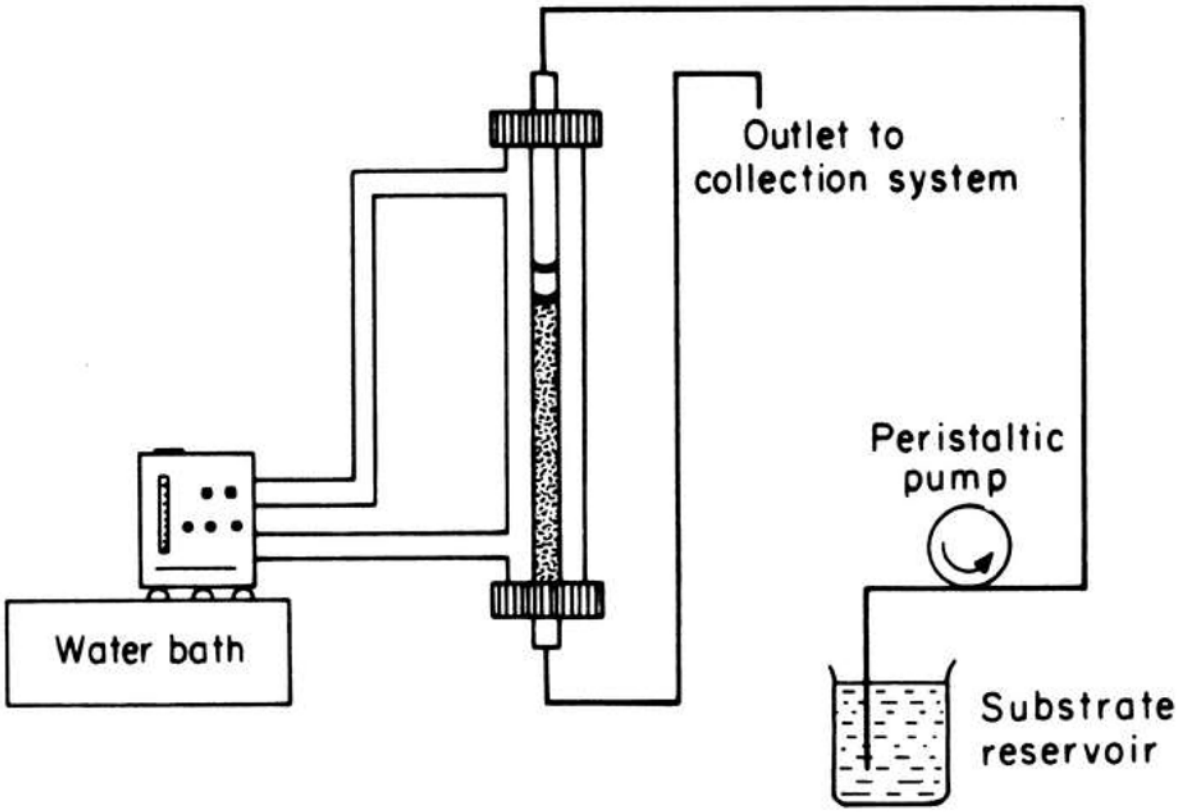

Figure 1. Column Assembly for Assay of Immobilized Glucose Isomerase.

Reagents and Solutions

Glucose substrate: Dissolve 539 g of anhydrous glucose and 1.0 g of magnesium sulfate ($MgSO_4 \cdot 7H_2O$) in 700 mL of water or the manufacturer's recommended buffer, previously heated to 50° to 60°. Cool the solution to room temperature, and adjust the pH as specified by the enzyme manufacturer. Transfer the solution to a 1000-mL volumetric flask, dilute to volume with water or the specified buffer, and mix. Transfer to a vacuum flask, and de-aerate for 30 min.

Magnesium sulfate solution: Dissolve 1.0 g of magnesium sulfate ($MgSO_4 \cdot 7H_2O$) in 700 mL of water. Adjust the pH to 7.5 to 8.0 as specified by the manufacturer, using 1 N sodium hydroxide, dilute to 1000 mL with water, and mix.

Sample preparation: Transfer to a 500-mL vacuum flask an amount of the sample, accurately weighed in grams or measured in milliliters, as appropriate, sufficient to obtain 2000 to 8000 glucose isomerase units (GI_cU). Add 200 mL of *Glucose substrate*, stir gently for 15 s, and repeat the stirring every 5 min for 40 min. De-aerate by vacuum for 30 min.

Column Preparation

Quantitatively transfer the *Sample preparation* to the column with the aid of *Magnesium sulfate solution* as necessary. Allow the enzyme granules to settle, and then place a porous disk so that it is even with, and in contact with, the top of the enzyme bed. Displace all of the air from the disk. Place a cotton plug about 1 or 2 cm above the disk. (This plug acts as a filter. It ensures proper heating of the solution and traps dissolved gases that may be present in the *Glucose substrate*.) Connect the tubing from the peristaltic pump with the top of the column, and seal the connection by suitable means to protect the column contents from the atmosphere. Place the inlet tube of the peristaltic pump into the *Glucose substrate* solution, and begin a downward flow of the *Glucose substrate* into the column at a rate of at least 80 mL/h. Maintain the flow rate for 1 h at room temperature.

Assay

Adjust the flow of the *Glucose substrate* to such a rate that a fractional conversion of 0.2 to 0.3 will be produced, based on the estimated activity of the sample. Calculate the fractional conversion from optical rotation values obtained on the starting *Glucose substrate* and the sample effluent, as specified under *Calculations*, below. After establishing the correct flow rate, run the column overnight (16 h minimum), then check the pH of the *Glucose substrate*, and readjust if necessary to the specified pH. Measure the flow rate, and collect a sample of the column effluent. Cover the effluent sample, allow it to stand for 30 min at room temperature, and then determine the fractional conversion of glucose to fructose (see *Calculations*, below). If the conversion is less than 0.2 or more than 0.3, adjust the flow rate to bring the conversion into this range. If a flow rate adjustment is required, collect an additional effluent sample after allowing the column to re-equilibrate for at least 2 h, and then determine the fractional conversion. Measure the flow rate, and collect an effluent sample. Cover the sample, let it stand at room temperature for 30 min, and determine the fractional conversion.

Calculations

Specific rotation: Measure the optical rotation of the effluent sample and of the starting *Glucose Substrate* at 25.0°, and calculate their specific rotations [see *Optical (Specific) Rotation, Appendix IIB*] by the equation:

$$[\alpha] = 100a/lpd$$

in which a is the corrected observed rotation, in degrees; l is the length of the polarimeter tube, in decimeters; p is the concentration of the test solution, expressed as grams of solute per 100 g of solution; and d is the specific gravity of the solution at 25°.

Fractional conversion: Calculate the fractional conversion, X, by the equation:

$$X = (a_E - a_S)/(a_F - a_S)$$

in which a_E is the specific rotation of the column effluent, a_S is the specific rotation of the *Glucose substrate*, and a_F is the specific rotation of fructose (which, in this case, has been calculated to be −94.54).

Activity: The enzyme activity is expressed in glucose isomerase units (GI_cU, the subscript c signifying column process). One GI_cU is defined as the amount of enzyme that converts glucose to fructose at an initial rate of 1 µmol/min, under the conditions specified.

Calculate the glucose isomerase activity by the equation:

$$GI_cU/g \text{ or } mL = (FS/W) \times X_E \times \ln[X_E/(X_E - X)]$$

in which F is the flow rate, in milliliters per min; S is the concentration of the *Glucose substrate*, in micromoles per milliliter; X is the fractional conversion, as determined above; X_E is the fractional conversion at equilibrium, or 0.51; and W is the weight or volume of the sample taken, in grams or milliliters, respectively.

- **Glucose Oxidase Activity**

Application and Principle: This procedure is used to determine glucose oxidase activity in preparations derived from *Aspergillus niger* var. The assay is based on the titrimetric measurement of gluconic acid produced in the presence of excess substrate and excess air.

Reagents and Solutions

Chloride–acetate buffer solution: Dissolve 2.92 g of sodium chloride and 4.10 g of sodium acetate in about 900 mL of water. Adjust the pH to 5.1 with either dilute acetic acid or dilute sodium hydroxide solution and dilute to 1000.0 mL.

Sodium hydroxide solution (0.1 N)

Hydrochloric acid solution (0.05 N): Standardized.

Phenolphthalein solution (2% w/v): Solution in methanol.

Octadecanol solution: Saturated solution in methanol.

Substrate solution: Dissolve 30.00 g of anhydrous glucose in 1000 mL of the *Chloride–acetate buffer solution*.

Sample preparation: Dissolve an accurately weighed amount of enzyme preparation in the *Chloride–acetate buffer solution*, and dilute in the buffer solution to obtain an enzyme activity of 5 to 7 activity units per milliliter.

Procedure

Transfer 25.0 mL of the *Substrate solution* to a 32- × 200-mm test tube. To a second 32- × 200-mm test tube transfer 25.0 mL of the *Chloride–Acetate buffer solution* (blank). Equilibrate both tubes in a 35° ± 0.1° water bath for 20 min. Add 3.0 mL of the *Sample preparation* to each test tube, mix, and insert a glass sparger into each tube with a preadjusted air flow of 700 to 750 mL/min. If excessive foaming occurs, add 3 drops of the *Octadecanol solution* to each tube. After exactly 15 min, remove the sparge and rinse any adhering reaction mixture back into the tube with water. Immediately add 10 mL of the *Sodium hydroxide solution* and 3 drops of the *Phenolphthalein solution* to each tube. Insert a small magnetic stirrer bar, stir, and titrate to the phenolphthalein endpoint with the standardized *0.05 N Hydrochloric acid solution*.

Calculation

One Glucose Oxidase Titrimetric unit of activity (GOTu) is the quantity of enzyme that will oxidize 3 mg of glucose to gluconic acid under the conditions of the assay. Determine the enzyme activity using the following equation:

$$GOTu/g = [(B - T) \times N \times 180 \times F]/[3 \times W]$$

in which *B* is the titration volume, in milliliters, of the blank; *T* is the titration volume, in milliliters, of the sample; *N* is the normality of the titrant; 180 is the molecular weight of glucose; *F* is the sample dilution factor; 3 is from the unit definition; and *W* is the weight, in grams, of the enzyme preparation contained in each milliliter of the sample solution.

- **Hemicellulase Activity**

Application and Principle: This procedure is used to determine hemicellulase activity of preparations derived from *Aspergillus niger* var. The test is based on the enzymatic hydrolysis of the interior glucosidic bonds of a defined locust (carob) bean gum substrate at 40° and pH 4.5. Determine the corresponding reduction in substrate viscosity with a calibrated viscometer.

Apparatus

Viscometer: Use a size 100 calibrated Cannon-Fenske Type Viscometer, or equivalent (Scientific Products, Catalog No. 2885-100).

Glass water bath: Use a constant-temperature glass water bath, maintained at 40° ± 0.1° (Scientific Products, Catalog No. W3520-10).

Stopwatches: Use two stopwatches—*Stopwatch No. 1*, calibrated in $^{1}/_{10}$ min for determining the reaction time (T_r), and *Stopwatch No. 2*, calibrated in ⅕ s for determining the efflux time (T_t).

Reagents and Solutions

Acetate buffer (pH 4.5)**:** Add 0.2 N sodium acetate, with continuous agitation, to 400 mL of 0.2 N acetic acid until the pH is 4.5 ± 0.05, as determined by a pH meter.

Locust bean gum: Use Powdered Type D-200 locust bean gum, or its equivalent (Meer Corp.). Because the substrate may vary from lot to lot, test each lot in parallel with a previous lot known to be satisfactory. Variations of more than ±5% viscosity in the average of a series of parallel tests indicate an unsuitable lot.

Substrate solution: Place 12.5 mL of 0.2 N hydrochloric acid and 250 mL of warm water (72° to 75°) in the bowl of a power blender (Waring two-speed, or equivalent, Scientific Products, Catalog No. 58350-1), and set the blender on low speed. Slowly disperse 2.0 g of *Locust Bean Gum*, on a moisture-free basis, into the bowl, taking care not to splash out any of the liquid in the bowl. Wash down the sides of the bowl with warm water, using a rubber policeman, cover the bowl, and blend at high speed for 5 min. Quantitatively transfer the mixture to a 1000-mL beaker, and cool to room temperature. Using a pH meter, adjust the mixture to pH 6.0 with 0.2 N sodium hydroxide. Quantitatively transfer the mixture to a 1000-mL volumetric flask, dilute to volume with water, and mix. Filter the substrate through gauze before use.

Sample preparation: Prepare a solution of the sample in water so that 1 mL of the final dilution will produce a change in relative fluidity between 0.18 and 0.22 in 5 min under the conditions specified in the *Procedure*.

Weigh the enzyme preparation, quantitatively transfer it to a glass mortar, and triturate with water. Quantitatively transfer the mixture to an appropriately sized volumetric flask, dilute to volume with water, and mix. Filter through Whatman No. 1 filter paper, or equivalent, before use.

Procedure

Scrupulously clean the viscometer by drawing a large volume of detergent solution, followed by water, through the instrument, and place the viscometer, previously calibrated, in the glass water bath in an exactly vertical position. Pipet 20.0 mL of *Substrate solution* and 4.0 mL of *Acetate buffer* into a 50-mL Erlenmeyer flask, allowing at least two flasks for each enzyme sample and one flask for a substrate blank. Stopper the flasks, and equilibrate them in the water bath for 15 min. At zero time, pipet 1.0 mL of the *Sample preparation* into the equilibrated substrate, start timing with stopwatch no. 1, and mix thoroughly. Immediately pipet 10.0 mL of this mixture into the

wide arm of the viscometer. After about 2 min, draw the reaction mixture above the upper mark into the driving fluid head by applying suction with a rubber tube connected to the narrow arm of the instrument. Measure the efflux time by allowing the reaction mixture to flow freely down past the upper mark. As the meniscus falls past the upper mark, start stopwatch no. 2, and at the same time, record the reaction time (T_R), in min, from stopwatch no. 1. As the meniscus of the reaction mixture falls past the lower mark, record the time (T_T), in seconds, from stopwatch no. 2. Immediately re-draw the reaction mixture above the upper mark and into the driving fluid head. As the meniscus falls freely past the upper mark, restart stopwatch no. 2, and at the same time record the reaction time (T_R), in min, from stopwatch no. 1. As the meniscus falls past the lower mark, record the time (T_T), in seconds, from stopwatch no. 2.

Repeat the latter operation, beginning with "Immediately re-draw the reaction mixture... ," until a total of four determinations is obtained over a reaction time (T_R) of NMT 15 min.

Prepare a substrate blank by pipetting 1.0 mL of water into a mixture of 20.0 mL of *Substrate solution* and 4.0 mL of *Acetate buffer*, and then immediately pipet 10.0 mL of this mixture into the wide arm of the viscometer. Determine the time (T_S), in seconds, required for the meniscus to fall between the two marks. Use an average of five determinations as T_S.

Prepare a water blank by pipetting 10.0 mL of water, previously equilibrated to 40° ± 0.1°, into the wide arm of the viscometer. Determine the time (T_W), in seconds, required for the meniscus to fall between the two marks. Use an average of five determinations as T_W.

Calculation

One hemicellulase unit (HCU) is defined as that activity that will produce a relative fluidity change of 1 over a period of 5 min in a locust bean gum substrate under the conditions specified. Calculate the relative fluidities (F_R) and T_N values (see definition below) for each of the four efflux times (T_T) and reaction times (T_R) as follows:

$$F_R = (T_S - T_W)/(T_T - T_W)$$

and

$$T_N = ½(T_T/60 \text{ s/min}) + T_R = (T_T/120) + T_R,$$

in which F_R is the relative fluidity for each reaction time; T_S is the average efflux time, in seconds, for the substrate blank; T_W is the average efflux time, in seconds, for the water blank; T_T is the efflux time, in seconds, of the sample reaction mixture; T_R is the elapsed time, in min, from zero time, that is, the time from addition of the enzyme solution to the buffered substrate until the beginning of the measurement of the efflux time (T_T); and T_N is the reaction time (T_R), in min, plus one-half of the efflux time (T_T) converted to min.

Plot the four relative fluidities (F_R) as the ordinate against the four reaction times (T_N) as the abscissa. This should result in a straight line. The slope of the line corresponds to the relative fluidity change per min and is proportional to the enzyme concentration. The slope of the best line through a series of experimental points is a better criterion of enzyme activity than is a single relative fluidity value. From the curve, determine the F_R values at 10 and 5 min. They should have a difference in fluidity of NMT 0.22 and NLT 0.18. Calculate the activity of the enzyme sample as follows:

$$\text{HCU/g} = 1000(F_{R10} - F_{R5}/W)$$

in which F_{R10} is the relative fluidity at 10 min reaction time; F_{R5} is the relative fluidity at 5 min reaction time; 1000 is milligrams per gram; and W is the weight, in milligrams, of the enzyme sample contained in the 1.0-mL aliquot of *Sample preparation* added to the equilibrated substrate in the *Procedure*.

- **Invertase Sumner Unit Activity**

Application and Principle: This procedure is used to measure the strength of invertase (sucrase) enzyme preparations from yeast *Saccharomyces* sp *(Kluyveromyces)* and *Saccharomyces* sp (*cerevisiae*). This assay is based on a 30-min hydrolysis of a 5.4% (w/v) solution of sucrose at pH 4.5 and 20°. The amount of monosaccharides produced is measured spectrophotometrically using a 3,5-Dinitrosalicylic Acid (DNS) acid–phenol reagent correlated to a glucose standard.

Reagents and Solutions

3,5-DNS acid stock solutions: Weigh 308 g of sodium potassium tartrate tetrahydrate and 19.4 g of sodium hydroxide into a 1000-mL volumetric flask. Dissolve in and dilute with deionized water to volume. In a second 1000-mL volumetric flask, transfer 10.7 g of 3,5-dinitrosalicylic acid. Dissolve in and dilute with deionized water to volume. In a third vessel (100-mL volumetric flask), transfer 8.33 g of phenol, 1.83 g of sodium hydroxide, and 8.33 g of sodium metabisulfite. Dissolve in and dilute with deionized water to volume.

3,5-DNS acid working solution: Combine the three solutions prepared for the *3,5-DNS acid stock solutions*, and set aside for at least 48 h. Pass the solution through a glass fiber filter. The final solution should be stored in a dark location in a plastic bottle. Re-filter the solution through a glass fiber filter when it becomes turbid.

pH 4.5 acetate buffer: Dissolve 29.25 g sodium acetate trihydrate in 300 mL of deionized water. Add 17.1 g of glacial acetic acid and mix. Adjust the pH to 4.50 with dilute sodium hydroxide or hydrochloric acid solutions as necessary. Quantitatively transfer the solution to a 500-mL volumetric flask and dilute with deionized water to volume.

Sucrose substrate solution (6.5% w/v)**:** Dissolve 16.25 g (dry wt basis) of sucrose in 200 mL deionized water and add 25.0 mL of *pH 4.5 acetate buffer*. Transfer the solution to a 250-mL volumetric flask and dilute with deionized water to volume. After the enzyme is added this will give a 5.4% solution. Prepare fresh daily.

Standard glucose solution (0.300%)**:** Dissolve 0.1500 g (dry wt basis) of D-(+)-glucose anhydrous in 40 mL of deionized water. Quantitatively transfer the solution to a 50-mL volumetric flask and dilute with deionized water to volume.

3,5-DNS working solution: Immediately prior to use, add 3.00 mL of the *Standard glucose solution (0.300%)* into 200 mL of the *3,5-DNS Acid Working Solution*. This is enough to assay three enzyme samples.

Enzyme sample preparation: Quantitatively dilute the enzyme in deionized water such that the final solution will contain 0.5 SU/mL. Solid samples that do not dissolve easily should be triturated with deionized water prior to transfer to an appropriate volumetric flask. Samples should be assayed within 30 min of dilution.

Analysis

Pipet 5 mL of the *Sucrose substrate solution* into a series of test tubes, allowing 6 test tubes for each enzyme sample (3 for the enzyme reaction and 3 for the enzyme blank). Also prepare 3 test tubes for the *Standard glucose solution* and 3 test tubes for the substrate blank, each containing 5 mL of the *Sucrose substrate solution*. Equilibrate these test tubes in a 20.0° ± 0.1° water bath for 10 min. At the same time, equilibrate 10 mL of each *Enzyme sample preparation* in a 20.0° ± 0.1° water bath for 10 min.

To start the enzyme reaction vessels, using a stopwatch beginning at time zero, in the order of the series and within regular time intervals, add 1 mL of the equilibrated *Enzyme sample preparation* into each of the three equilibrated *Sucrose substrate solution* test tubes and mix thoroughly. Prepare enzyme blanks by placing an amount of the *Enzyme sample preparation* in a boiling water bath for 10 min and cooling in an ice bath for 5 min. Add 1 mL of this deactivated enzyme solution

to each of the three appropriate *Sucrose Substrate Solution* test tubes, and mix thoroughly.
To prepare the glucose standards, add 1 mL of the *Standard sucrose solution* to each of the 3 appropriate *Sucrose substrate solution* test tubes.
To prepare the substrate blanks, add 1 mL of deionized water to each of the 3 appropriate *Sucrose substrate solution* test tubes.
At time equals exactly 30 min, in the order of the series and within the regular time intervals, stop the reaction by pipetting 3 mL of the appropriate enzyme reaction mixture into a test tube (>50 mL capacity) containing 7 mL of the *3,5-DNS working solution* and mix thoroughly. Likewise, transfer 3 mL of each glucose standard solution, 3 mL of each substrate blank solution, and 3 mL of each of enzyme blank solution into the appropriate test tubes (>50 ml capacity) containing 7 mL of *3,5-DNS working solution,* and mix thoroughly.
Place all test tubes into a boiling water bath for exactly 10 min. At the end of 10 min, place all test tubes in an ice water bath for 5 min. To each test tube, add 40 mL deionized water and mix thoroughly. Allow the test tubes to set at room temperature for at least 10 min and then measure the absorbance at 515 nm against a water blank in a 1-cm cuvette.

Calculation

One Sumner Unit (SU) is the quantity of enzyme which, under the conditions of the assay, will convert 1 mg of sucrose to glucose and fructose in 5 min. Calculate the activity of the enzyme preparation as follows:

$$\text{SU/g} = [(A_U - A_B)/(A_S - A_W)] \times (0.5/C)$$

in which A_U is the average absorbance of the enzyme sample; A_B is the average absorbance of the enzyme blank; A_S is the average absorbance of the glucose standard; A_W is the average absorbance of the substrate blank; C is the concentration (in g/mL) of the *Enzyme sample preparation;* and 0.5 = [(3 mg glucose × 5 min unit definition)/30 min reaction].

- **Lactase Neutral (β-Galactosidase) Activity**

Application and Principle: This procedure is used to determine the neutral lactase activity of enzyme preparations derived from *Kluyveromyces marxianus* var. *lactis* and *Saccharomyces* sp. The assay is based on a 10-min hydrolysis of an *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate at 30.0° ± 0.1° and at pH 6.50.

Reagents and Solutions

Magnesium solution: Dilute 24.65 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in about 950 mL of water. Transfer the solution into a 1000-mL volumetric flask, dilute to volume with water, and mix.

EDTA solution: Dissolve 1.86 g of disodium EDTA dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) in about 950 mL of water. Transfer the solution into a 1000-mL volumetric flask, dilute to volume with water, and mix.

P-E-M buffer: Dissolve 8.8 g of potassium dihydrogen phosphate (KH_2PO_4) and 8.0 g of dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$) in about 900 mL of water. Add 10.0 mL of *Magnesium solution* and 10.0 mL of *EDTA solution*. Transfer the solution into a 1000-mL volumetric flask, dilute to volume with water, and mix. The pH should be 6.50 ± 0.05.

Lactase reference preparation: (Highly concentrated lactase preparation) This preparation can be obtained from Gist-Brocades, Delft, The Netherlands.

ONPG: (*o*-nitrophenyl-β-D-galactopyranoside) is validated according to the following procedure:

Validation of New ONPG

Transfer 150, 250, and 375 mg of the new *ONPG* into separate 100-mL volumetric flasks, dilute to volume with *P-E-M buffer*, and mix. Prepare solutions of the *Lactase reference*

preparation by weighing an amount of *Lactase reference preparation* corresponding to 5000 ± 250 Neutral Lactase Units (NLU) accurately to within 1 mg in duplicate in 50-mL volumetric flasks, dissolve in *P-E-M buffer*, dilute to volume with the same, and mix. Prepare dilutions of this initial solution with *P-E-M buffer* so that 1 mL of the final dilution will contain 0.0375, 0.0750, and 0.1125 NLU of activity. In duplicate, determine the enzyme activity of the three enzyme concentrations using each of the new *ONPG Substrate* solutions corresponding to 150, 250, and 375 mg and the old *ONPG substrate* at 250 mg by following the steps in the *Procedure*, below.

Calculation

Calculate the enzyme activity following the steps indicated under *Calculation for NLU Activity*, below. Determine the average of the duplicates for each enzyme concentration at each level of *ONPG substrate* (the maximum allowable difference between these duplicates is 6.5%). Determine the overall average for the three enzyme concentrations (0.0375, 0.0750, and 0.1125) for each *ONPG substrate* level (150, 250, and 375 mg of *ONPG*).

To determine the overall average of three enzyme concentrations at 150 mg of *ONPG*:

$$X = (A + B + C)/3,$$

in which A is the average result of 0.0375 at 150 mg of *ONPG*, B is the average result of 0.0750 at 150 mg of *ONPG*, and C is the average result of 0.1125 at 150 mg of *ONPG*.

To determine the overall average of three enzyme concentrations at 250 mg of *ONPG*:

$$Y = (D + E + F)/3,$$

in which D is the average result of 0.0375 at 250 mg of *ONPG*, E is the average result of 0.0750 at 250 mg of *ONPG*, and F is the average result of 0.1125 at 250 mg of *ONPG*.

To determine the overall average of three enzyme concentrations at 375 mg of *ONPG*:

$$Z = (G + H + I)/3,$$

in which G is the average result of 0.0375 at 375 mg of *ONPG*, H is the average result of 0.0750 at 375 mg of *ONPG*, and I is the average result of 0.1125 at 375 mg of *ONPG*.

The *ONPG* analyzed is suitable for use when the following specifications are met for each *ONPG* concentration:

1. The average result of each enzyme concentration for each *ONPG* level does not deviate more than 3% from the overall average of the three enzyme concentrations for that level of *ONPG*. For example, *A* or *B* or *C* should not deviate more than 3% from *X*; *D* or *E* or *F* should not deviate more than 3% from *Y*; *G* or *H* or *I* should not deviate more than 3% from *Z*.
2. The overall average of the three enzyme concentrations found for 150 mg of *ONPG* (*X*) should not vary more than 81% to 99% of the overall average of the three enzymes concentrations found for 250 mg of *ONPG* (*Y*). The overall average of the three enzyme concentrations found for 375 mg of *ONPG* (*Z*) should not vary more than 96% to 114% of the overall average of the three enzyme concentrations of 250 mg of *ONPG* (*Y*).
3. The absorbance of each blank is less than 0.050.
4. For each new lot of *ONPG*, the overall average of the three enzyme concentrations found for 250 mg of *ONPG* (*Y*) per 100 mL should be within 5% of the overall average of the three enzyme concentrations found for 250 mg of *ONPG* of the lot in use at that moment.

ONPG substrate: Dissolve 250.0 mg *ONPG* (use lot currently in use) in about 80 mL of *P-E-M buffer*. Transfer the solution to a 100-mL volumetric flask, dilute to volume with *P-E-M buffer*, and mix. Prepare, at most, 2 h before incubation.

Sodium carbonate solution: Dissolve 50.0 g of sodium carbonate anhydrous (Na_2CO_3) and 37.2 g of disodium EDTA dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) in about 900 mL of water. Transfer the solution into a 1000-mL volumetric flask, dilute to volume with water, and mix.

Standard *o*-nitrophenol solution: Transfer 139.0 mg of *o*-nitrophenol into a 1000-mL volumetric flask, dissolve in 10 mL of 96% ethanol, dilute to volume with water, and mix. Pipet

2-, 4-, 6-, 8-, 10-, 12-, and 14-mL portions of this solution into a series of 100-mL volumetric flasks, add 25 mL of *Sodium carbonate solution* to each, dilute each to volume with *P-E-M buffer*, and mix. The dilutions contain, respectively, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12 and 0.14 µmol/mL of *o*-nitrophenol.

Determine the absorbance of each dilution at 420 nm in a 1-cm path-length cell, with a suitable spectrophotometer, using water as the blank. For each dilution, plot absorbance against µmol of *o*-nitrophenol (this must result in a straight line through the origin). Divide the absorbance of each dilution by µmol of *o*-nitrophenol to obtain the extinction coefficient (*M*) at that dilution (the slope of the line is the extinction coefficient). Average the seven values thus calculated (this should result in a value of 4.60 ± 0.05).

Test preparation: Using a volumetric flask, prepare a test solution from the starting enzyme preparation by accurately weighing out a minimum of 1 g of sample to the nearest milligram. Dissolve in *P-E-M buffer* so that 1 mL of the final dilution will contain between 0.027 and 0.095 NLU. Transfer 1 mL of this final dilution to a 15- × 150-mm test tube as the *Test preparation*. Perform in duplicate.

Procedure

Equilibrate the test tubes containing each *Test preparation* in a water bath maintained at 30.0° ± 0.1° for at least 5 but NMT 15 min. At zero time, in the order of the series and at regular time intervals, rapidly pipet 5.00 mL of *ONPG substrate*, equilibrated at 30.0° ± 0.1°, into the test tubes, and mix by shaking. After a 10.0-min incubation (reaction) time, in the same order and with the same regular intervals, pipet 2.00 mL of *Sodium carbonate solution* into each, mix by shaking, and hold at room temperature. Determine the absorbance of each solution within 30 min at 420 nm in a 1-cm cell with a suitable spectrophotometer, using as the blank a solution prepared in the same manner as for the sample except adding *ONPG substrate* and *Sodium carbonate solution* in reverse order.

Calculation for NLU Activity

One Neutral Lactase Unit (NLU) is defined as that quantity of enzyme that will liberate 1.30 µmol/min of *o*-nitrophenol under the conditions of the assay. Calculate the activity of the enzyme preparation taken for the analysis as follows:

$$\text{NLU/g} = [(A \times 8 \times F)/(M \times 10 \times W)]/1.30$$

in which *A* is the average of the absorbance readings for the sample, corrected for the sample blank; 8 is the volume, in milliliters, of the incubation mixture after termination; *F* is the total dilution factor of the sample; *M* is the extinction coefficient, determined as directed under *Standard o-nitrophenol solution*; 10 is the incubation time, in min; *W* is the sample weight, in grams; and 1.30 is the factor used in the unit definition.

- **Lactase (Acid) (β-Galactosidase) Activity**

Application and Principle: This procedure is used to determine lactase activity of enzyme preparations derived from *Aspergillus oryzae* var. The assay is based on a 15-min hydrolysis of an *o*-nitrophenyl-β-D-galactopyranoside substrate at 37° and pH 4.5.

Reagents and Solutions

2.0 N Acetic acid: Dilute 57.5 mL of glacial acetic acid to 500 mL with water. Mix well, and store in a refrigerator.

4.0 N Sodium hydroxide: Dissolve 40.0 g of sodium hydroxide in sufficient water to make 250 mL.

Acetate buffer: Combine 50 mL of *2.0 N Acetic acid* and 11.3 mL of *4.0 N Sodium hydroxide* in a 1000-mL volumetric flask, and dilute to volume with water. Verify that the pH is 4.50 ± 0.05, using a pH meter, and adjust, if necessary, with *2.0 N Acetic acid* or *4.0 N Sodium hydroxide*.

2.0 mM *o*-Nitrophenol stock: Transfer 139.0 mg of *o*-nitrophenol to a 500-mL volumetric flask, dissolve in 10 mL of USP alcohol (95% ethanol) by swirling, and dilute to volume with 1% sodium carbonate.

***o*-Nitrophenol standards**

0.10 mM Standard solution: Pipet 5.0 mL of the *2.0 mM o-Nitrophenol stock* solution into a 100-mL volumetric flask, and dilute to volume with 1% sodium carbonate solution.

0.14 mM Standard solution: Pipet 7.0 mL of the *2.0 mM o-Nitrophenol stock* solution into a 100-mL volumetric flask, and dilute to volume with 1% sodium carbonate solution.

0.18 mM Standard solution: Pipet 9.0 mL of the *2.0 mM o-Nitrophenol stock* solution into a 100-mL volumetric flask, and dilute to volume with 1% sodium carbonate solution.

Substrate: Transfer 370.0 mg of *o*-nitrophenyl-β-D-galactopyranoside to a 100-mL volumetric flask, and add 50 mL of *Acetate buffer*. Swirl to dissolve, and dilute to volume with *Acetate buffer*.

[NOTE—Perform the assay procedure within 2 h of *Substrate* preparation.]

Test preparation: Prepare a solution from the test sample preparation such that 1 mL of the final dilution will contain between 0.15 and 0.65 lactase unit. Weigh, and quantitatively transfer the enzyme to a volumetric flask of appropriate size. Dissolve the enzyme in water, swirling gently, and dilute with water if necessary.

[NOTE—Perform the assay procedure within 2 h of dissolution of the *Test preparation*.]

System suitability: Determine the absorbance of the three o-*Nitrophenol standards* at 420 nm in a 1-cm cell, using a suitable spectrophotometer. Use water to zero the instrument. Calculate the millimolar extinction, *M*, for each of the o-*Nitrophenol standards* (0.10, 0.14, and 0.18 mM) by the equation

$$\varepsilon = A_n/C$$

in which A_n is the absorbance of each o-*Nitrophenol standard* at 420 nm and *C* is the corresponding concentration of *o*-nitrophenol in the standard. *M* for each standard should be approximately 4.60/mM. Perform a linear regression analysis of the absorbance readings of the three o-*Nitrophenol standards* versus the *o*-nitrophenol concentration in each (0.10, 0.14, and 0.18 mM). The r^2 should not be less than 0.99. Determine the mean *M* of the three o-*Nitrophenol standards* for use in the calculations below.

Procedure

For each sample or blank, pipet 2.0 mL of the *Substrate* solution into a 25- × 150-mm test tube, and equilibrate in a water bath maintained at 37.0° ± 0.1° for approximately 10 min. At zero time, rapidly pipet 0.5 mL of the *Test preparation* (or 0.5 mL of water as a blank) into the equilibrated substrate, mix by brief (1 s) vortex, and immediately return the tubes to the water bath. After exactly 15 min of incubation, rapidly add 2.5 mL of 10% sodium carbonate solution, and vortex the tube to stop the enzyme reaction. Dilute the samples and blanks to 25.0 mL by adding 20.0 mL of water, and thoroughly mix. Determine the absorbance of the diluted samples and blanks at 420 nm in a 1-cm cell, using a suitable spectrophotometer. Use water to zero the instrument.

Calculation

One lactase unit (ALU) is defined as that quantity of enzyme that will liberate *o*-nitrophenol at a rate of 1 µmol/min under the conditions of the assay.

Calculate the activity (lactase activity per gram) of the enzyme preparation taken for analysis as follows:

$$\text{ALU/g} = [(A_S - B)(25)]/[(\varepsilon)(15)(W)]$$

in which A_S is the average of absorbance readings for the *Test preparation*; *B* is the average of absorbance readings for the blank; 25 is the final volume, in milliliters, of the diluted incubation mixture; *ε* is the mean absorptivity of the o-*Nitrophenol standards* per micromole, 15 is the incubation time, in min, and *W* is the weight, in grams, of original enzyme preparation contained in the 0.5-mL aliquot of *Test preparation* used in the incubation.

- **Lipase Activity**

Application and Principle: This procedure is used to determine the lipase activity in preparations derived from microbial sources and animal pancreatic tissues. The assay is based on the potentiometric measurement of the rate at which the preparations will catalyze the hydrolysis of tributyrin.

Apparatus

Automatic recording titrimeter: Use an instrument operating in the pH stat mode and equipped with a jacketed titration cell (Radiometer Titralab, or equivalent).

Constant temperature bath: Operated at 30° ± 0.1°.

Blender

Reagents and Solutions

0.05 N sodium hydroxide: Dissolve 2.0 g of sodium hydroxide in water, and dilute to 100 mL. Standardize with NIST grade potassium hydrogen phthalate.

Emulsification reagent: Dissolve 17.9 g of sodium chloride and 0.41 g of monobasic potassium phosphate in about 400 mL of water. Add 540 mL of glycerol and, with vigorous stirring, add 6.0 g of gum arabic (Sigma, Catalog No. G 9752). Stir until dissolved. Dilute to 1000 mL.

Glycine buffer (0.1 M): Dissolve 7.50 g of glycine (Sigma, Catalog No. G 126) and 3.8 g of sodium hydroxide in about 900 mL water. Adjust the pH to 10.8, and dilute to 1000 mL.

[Note—Instead of the *Glycine buffer*, some enzyme preparations may require the use of 0.01 M pH 8.0 *Tris buffer* prepared as directed for *Tris Buffer* under *Proteolytic Activity, Bacterial (PC)*, except to titrate with 1 N hydrochloric acid to pH 8.0.]

Substrate emulsion: Transfer 15.9 mL of tributyrin (Sigma, Catalog No. T 8626) to a blender, add 50 mL *Emulsification reagent* and 235 mL water. Blend for 15 min at maximum speed. Equilibrate in the 30° constant temperature bath for at least 15 min before use. Use within 4 h.

Sample preparation: Dissolve an accurately weighed amount of the enzyme preparation in *Glycine buffer* (or pH 8.0 *Tris buffer* if specified) so that each milliliter contains between 2000 and 5000 lipase units per milliliter. Accurately dilute a portion of this solution with water to obtain a final solution containing between 0.5 and 1.5 lipase units per milliliter.

Procedure

Fill the titrator buret with the *0.05 N sodium hydroxide* solution, and following the manufacturer's instructions, set the temperature to 30° and the pH set point to 7.0. Transfer 15.0 mL of the *Substrate emulsion* to the titration cell, and add a small stirrer bar. Add 1.0 mL of the diluted *Sample preparation*, and actuate the titrator. Record the rate of *0.05 N sodium hydroxide* addition. Stop the titration after a constant (linear) rate of addition has been observed for 5 min. Determine the addition rate, in milliliters per min, from the linear portion of the recording and record this value as *R*.

Calculation

One lipase unit (LU) is defined as the quantity of enzyme that will liberate 1 µmol of butyric acid per min under the conditions of the test.

Calculate the activity of the enzyme preparation by the formula:

$$\text{LU/g} = R \times N \times 1000/W$$

in which *R* is the addition rate, in milliliters per min; *N* is the normality of the *Sodium hydroxide* solution; 1000 converts mM to µM; and *W* is the weight, in grams, of the enzyme preparation contained in 1 mL of the diluted *Sample preparation*.

- **Lipase (Microbial) Activity for Medium- and Long-Chain Fatty Acids**

Application and Principle: This procedure is used to determine the lipase activity in preparations derived from microbial sources. The assay is based on the measurement of the amount of free fatty acids formed from an olive oil emulsion in the presence of sodium taurocholate over a fixed time interval. This assay is particularly used for measuring lipase activity in foods.

Reagents and Solutions

Gum arabic solution: Dissolve 110 g of gum arabic (acacia) (Sigma, Catalog No. G-9752, or equivalent) and 12.5 g of analytical-grade calcium chloride ($CaCl_2 \cdot 2H_2O$) in 800 mL of water in a 1000-mL volumetric flask, and dilute to volume with water. Shake or stir for 30 min at room temperature to dissolve completely. Centrifuge at 4000 × g for 20 min or filter through a Büchner funnel using Celite as a filter aid. Store the supernatant or filtrate at 4°. Divide into single-use, 24-mL aliquots. The solution is stable for 6 months at −20°.

Substrate emulsion: Place 130 mL of olive oil (Sigma, Catalog No. O-1500, or equivalent) and 400 mL of *Gum arabic solution* in a mixer bowl, and cool the mixture to 5° to 10° on ice. Emulsify the mixture with a Waring Blender, or equivalent, operated at high speed for 30 min, keeping the temperature below 30° by repeatedly mixing at high speed for 5 min and turning the blender off for 1 min. Check the quality of the emulsion microscopically: 90% of the droplets should have a diameter equal to or less than 3 µm, and the remaining 10% should not exceed 10 µm. The emulsion is stable for 3 days at 4°.

Reference standard solution: Dissolve an aliquot of Fungi Lipase-International FIP Standard (International Commission on Pharmaceutical Enzymes F.I.P., Center for Standards of the Federation Internationale Pharmaceutique, Harelbekestraat 72, B-9000 Gent, Belgium) in a 1% sodium chloride solution and dilute it to obtain a solution of 2.4 to 3.6 FIP microbial lipase units per milliliter. Prepare this solution fresh.

0.02 N Sodium hydroxide solution: Prepare daily by diluting 10 mL of analytical-grade 1 N sodium hydroxide to 500 mL with recently boiled water.

0.5% Sodium taurocholate solution: Dissolve 0.5 g of sodium taurocholate (DIFCO, Catalog No. 0278-15-8) in 100 mL of water. Prepare this solution fresh.

Sample preparation: Dissolve an accurately weighed amount of the enzyme preparation in a 1% sodium chloride solution, and dilute to obtain a solution of 2.4 to 3.6 FIP microbial lipase units per milliliter. Prepare this solution fresh.

Procedure

[Note—Assay the Fungi Lipase-International FIP Standard as an internal standard each time.]

Automatic titration: Use an automatic titration device with a 25 mL ± 0.02 mL buret, a pH meter giving a resolution to 0.01, and a reaction vessel with a capacity of 100 mL. Add 24 mL of *Substrate emulsion*, 9 mL of water, and 2 mL of *0.5% Sodium taurocholate solution* to the reaction vessel. Place the reaction vessel in a water bath preheated to 37° ± 0.5° over a hot plate provided with magnetic stirring, and add a magnet to the reaction vessel. Pre-incubate the reaction vessel at 37° ± 0.5° for 10 to 15 min while stirring at about 300 rpm. Immerse a pH-electrode and the tip of the buret into the solution. If desired, gently blow nitrogen gas onto the solution. Adjust the pH of the solution to 7.0 with *0.02 N Sodium Hydroxide Solution*. Set the automatic buret to zero. Add 5.0 mL of the enzyme solution while simultaneously starting a

timer. Maintain the pH at 7.0 by automatic titration. After 10.0 min, abruptly (within 30 s) bring the pH to 9.0 by manually adding additional *0.02 N Sodium hydroxide solution*. Record the volume of *0.02 N Sodium hydroxide solution* consumed as N_1. Run the test with a blank by setting up the titration in the same manner, except after adjusting the pH to 7.0 with *0.02 N Sodium hydroxide solution*, set the automatic buret to zero, and maintain the pH at 7.0 by automatic titration. After 10.0 min, abruptly (within 30 s) bring the pH to 9.0 as before, and then add 5.0 mL of enzyme solution. Because the enzyme lowers the pH, return the pH to 9.0 by adding *0.02 N Sodium hydroxide solution*. Record the volume of *0.02 N Sodium hydroxide solution* consumed as N_2.

Manual titration: Follow the same procedure as with *Automatic titration*, but keep the pH at 7.0 with *0.02 N Sodium hydroxide solution* from a 25-mL buret, demarked in 0.02-mL units.

Calculation

One unit of enzyme activity (FIP Unit) is defined as that quantity of a standard lipase preparation (Fungi Lipase-International FIP Standard) that liberates the equivalent of 1 µmol of fatty acid per min from the *Substrate emulsion* under the described assay conditions. The specific activity is expressed in international FIP units per milligram of the *Sample preparation*.

The use of an enzyme reference standard of known activity, controlled by the Center for Standards of the Commission, eliminates difficulties from interlaboratory differences in quality of reagents such as the *Gum arabic solution*, olive oil, or *Substrate emulsion* or in the set-up of the experiment. The activity (FIP U/mg) using an enzyme reference standard is calculated by the formula:

$$\text{Result} = (A \times C)/B$$

in which A is the specific activity, in units/mg, of the test sample (measured); B is the specific activity, in units/mg, of Fungi Lipase-International FIP Standard (measured); and C is the number of FIP units/mg of Fungi Lipase-International FIP Standard as indicated on the container.

One milliliter of the *0.02 N Sodium hydroxide solution* corresponds with the neutralization of 20 µmol of fatty acids. Five milliliters of enzyme solution liberates $(N_1 - N_2)$ mL × 20 µmol of fatty acids over a 10-min time interval. If the enzyme solution contains W mg of enzyme preparation per milliliter, the specific activity, in units/mg, is calculated as follows:

$$\text{Result} = [(N_1 - N_2) \times 20]/(10 \times 5 \times W)$$

in which $(N_1 - N_2)$ is the volume, in milliliters, of the *0.02 N Sodium hydroxide solution* used for the titration.

- **Lysozyme Activity[1]**

Application and Principle: The purpose of this procedure is to determine the lysozyme activity in purified lysozyme preparations derived from animal or microbial sources. The assay is based on the rate of decrease in absorbance at 450 nm, attributed to the lysis of *Micrococcus lysodeikticus* by lysozyme. The decrease in absorbance is measured using a UV/V spectrophotometer equipped to control the sample temperature at 25°.

[Note—Ensure that all glassware and supplies are heat sterilized. The work area should be aseptically clean. Any residual lysozyme contamination will adversely affect the results of the assay.]

Reagents and Solutions

Sodium phosphate buffer solution: Dissolve 10.4 g of monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) in 500 mL of sterile, deionized water in a 1000-mL volumetric flask, and dilute to volume. Similarly, dissolve 9.465 g of anhydrous dibasic sodium phosphate (Na_2HPO_4) in sterile, deionized water, and dilute to 1000 mL. Mix 815 mL of the monobasic sodium phosphate

solution with 185 mL of the dibasic sodium phosphate solution. Adjust the pH of the buffer to 6.2; when checking the pH, use an aliquot of the buffer to prevent contamination of the solution. Adjust the pH by adding more monobasic or dibasic sodium phosphate solution as needed. The buffer solution may be stored under refrigeration for up to 1 month.

Substrate solution: Add 30 to 40 mg of *Micrococcus lysodeikticus* (Sigma M-3770, or equivalent) to 100 mL of *Sodium phosphate buffer solution* in a 250-mL Erlenmeyer flask, tilt gently to mix, and do not shake. Allow the substrate to incubate at 37° for 30 min before using it. The substrate solution is stable for 2 h at room temperature. Zero a spectrophotometer against air, then measure the absorbance of the substrate solution, which should give a reading of 1.7 ± 0.1 at 450 nm.

[NOTE—If the absorbance is significantly lower than 1.7, do not adjust the concentration. Run the analysis, and check the rate of the reaction. The rate of the decrease in absorbance should range between 0.03 and 0.06 units per min.]

Standard preparation: Use a commercial reference standard lysozyme of a specified strength from an animal or microbial source in accordance with the origin of the preparation being measured. Measure 50 mg of the reference standard lysozyme into a 50-mL volumetric flask, and dissolve, with stirring, in approximately 25-mL of *Sodium phosphate buffer solution*. Dilute to volume with *Sodium phosphate buffer solution*, and mix thoroughly. If desired, freeze aliquots of this *Standard preparation* for subsequent assays. Quantitatively transfer 3 mL of the *Standard preparation* to a 100-mL volumetric flask, and dilute to volume with *Sodium phosphate buffer solution*.

Sample preparation: Measure 50 mg of sample into a 50-mL volumetric flask. Dissolve the sample, with stirring, in approximately 25 mL of *Sodium phosphate buffer solution*. Dilute to volume with *Sodium phosphate buffer solution*, and mix the solution thoroughly. Quantitatively transfer 3 mL of the solution to a 100-mL volumetric flask, and dilute to volume with *Sodium phosphate buffer solution*.

Procedure

Conduct the test in a spectrophotometer equipped to maintain a temperature of 25° in the cell compartment. Perform the test in triplicate for the *Standard preparation* and for the *Sample preparation*.

Place a 1-cm cell into the spectrophotometer, and adjust the absorbance to zero. Pipet 2.9 mL of *Substrate solution* into the cell; the initial absorbance of the solution should be 1.7 ± 0.1 at 450 nm (see *Note* above). Pipet 0.1 mL of the *Standard preparation* into the substrate, and mix well. Record the decrease in absorbance over 3 min, recording the absorbance value approximately every 15 s. The rate of the decrease in absorbance should be linear, and range between 0.03 and 0.06 per min. Repeat the procedure with the *Sample preparation*.

Calculation

One lysozyme unit is defined as the amount of lysozyme that causes a decrease in absorbance of 0.001 per min at 450 nm, 25°, and pH 6.2, using a suspension of *Micrococcus lysodeikticus* as the substrate.

The assay stabilizes over the first min; disregard the first min of readings in the calculation. Determine the average absorbance change per min using only the linear portion of the curve where the rate of change is constant, usually the final 2 min.

Calculate the number of lysozyme units per mg by the equation:

$$\text{lysozyme units} = (A_1 - A_2)/(T \times W \times 0.001)$$

in which A_1 is the initial absorbance reading in the straight-line portion of the curve; A_2 is the final absorbance reading in the straight-line portion of the curve; T is the elapsed time, in min,

between the initial and final absorbance readings; *W* is the weight, in mg, of the lysozyme in the volume of *Sample preparation* used in the *Assay*; and 0.001 is the decrease in absorbance caused by one unit of lysozyme per min.

- **Maltogenic Amylase Activity**

Application and Principle: This procedure is used to determine maltogenic amylase activity in preparations derived from *Bacillus subtilis* containing a *Bacillus stearothermophilus* amylase gene. The test is based on a 30-min hydrolysis of maltotriose under controlled conditions and measurement of the glucose formed by high-performance liquid chromatography (HPLC).

Reagents and Solutions

Citrate buffer, 0.1 M: Dissolve 5.255 g of citric acid ($C_6H_8O_7 \cdot H_2O$) in about 150 mL of water. Adjust the pH to 5.0 with 1 N sodium hydroxide, and dilute to 250 mL.

Substrate solution: Dissolve 1.00 g of maltotriose (Sigma Chemical Co., Catalog No. M 8378) in *Citrate buffer* in a 50-mL volumetric flask, and dilute to volume with *Citrate buffer*.

Sodium chloride solution, 1 M: Dissolve 29.22 g of sodium chloride in water, and dilute to 500 mL.

Amberlite MB-1 ion exchange resin: Air dry at room temperature for about 1 week. Protect from contamination.

Glucose standards: Dissolve 1.80 g of anhydrous glucose in water, and dilute to 1000 mL. Transfer 20.0, 50.0, 75.0, and 100.0 mL to separate 100-mL volumetric flasks, and dilute to volume with water. These solutions contain 0.36, 0.9, 1.35, and 1.80 mg of glucose per mL. Using filtered, degassed water as the mobile phase, equilibrate an HPX 87C column, or equivalent, in a high-performance liquid chromatograph equipped with a differential refractometer. Chromatograph 5-µL portions of the glucose standards, and record the chromatograms. Prepare a standard curve of the glucose concentration versus the peak height.

Sample preparation: Prepare a solution of each sample to contain approximately 7.5 Maltogenic Amylase Units (MANU) per mL. Further dilute an aliquot of each sample so that the final dilution contains 1% by volume of the *Sodium chloride solution, 1 M* and contains between 0.150 and 0.600 MANU per mL.

Procedure

Transfer 2.00 mL of each sample to separate test tubes, and equilibrate in the 37° water bath for at least 10 min. At the same time, equilibrate the *Substrate solution* in the same water bath. At zero time, transfer 2.0 mL of the equilibrated *Substrate solution* to the first sample tube, mix thoroughly, and return the tube to the 37° bath. Repeat the process for each sample. After exactly 30.0 min, transfer the test tube to a boiling water bath for 15 min, then remove and cool to room temperature. Add approximately 100 mg of *Amberlite MB-1 ion exchange resin* to each tube, place the tubes on the shaker, and mix for at least 15 min. Pass the treated solution through a 0.45-µm filter. Use a separate filter for each sample. Inject a 5-µL portion of each filtered sample into a previously equilibrated high-performance liquid chromatograph equipped with an HPX 87C column (Biorad, or equivalent) and a differential refractometer. Filtered, degassed water is the mobile phase. Record the elution curve.

Calculation

One Maltogenic Amylase Unit (MANU) is defined as the amount of enzyme that will cleave maltotriose at a rate of 1 µmol/min under the conditions of the test. From the elution curve of each sample, determine the glucose concentration (*G*) in the sample from the previously prepared standard curve. Calculate the MANU/g by the equation:

$$\text{MANU/g} = G \times 4 \times F/180.1 \times 30 \times W$$

in which *G* is the glucose concentration in the test solution; 4 is the total test solution volume; 30 is the reaction time, in min; *F* is the dilution factor; and *W* is the sample weight, in g.

- **Milk-Clotting Activity**

Application and Principle: This procedure is to be applied to enzyme preparations derived from either animal or microbial sources.

Apparatus

Bottle-rotating apparatus: Use a suitable assembly, designed to rotate at a rate of 16 to 18 rpm.

Sample bottles: Use 125-mL, squat, round, wide-mouth bottles (such as Scientific Products, Catalog No. B-7545-125).

Substrate solution: Dissolve 60 g of low-heat, nonfat dry milk (such as Galloway West, Peake Grade A) in 500 mL of a solution, adjusted to pH 6.3 if necessary, containing in each mL 2.05 mg of sodium acetate ($NaC_2H_3O_2$) and 1.11 mg of calcium chloride ($CaCl_2$).

Standard preparation: Use a standard-strength rennet, bovine rennet, microbial rennet (*Endothia parasitica*), or microbial rennet (*Mucor* species), as appropriate for the preparation to be assayed. Such standards, which are available from commercial coagulant manufacturers, should be of known activity. Dilute the standard-strength material 1 to 200 with water, and mix. Equilibrate to 300 before use, and prepare no more than 2 h before use.

Sample preparation: Prepare aqueous solutions or dilutions of the sample to produce a final concentration such that the clotting time, as determined in the *Procedure* below, will be within 1 min of that of the *Standard preparation*. Prepare no more than 1 h before use.

Procedure

Transfer 50.0 mL of the *Substrate solution* into each of four 125-mL *Sample bottles*. Place the bottles on the *Bottle-rotating apparatus*, and suspend the apparatus in a water bath, maintained at 30° ± 0.5°, so that the bottles are at an angle of approximately 20° to 30° to the horizontal. Immerse the bottles so that the water level in the bath is about equal to the substrate level in the bottles. Begin rotating the apparatus at 16 to 18 rpm, then add 1.0 mL of the *Sample preparation* to each of two bottles, and record the exact time of addition. Add 1.0 mL of the *Standard preparation* to each of the other two bottles, recording the exact time.

Observe the rotating bottles, and record the exact time of the first evidence of clotting (i.e., when fine granules or flecks adhere to the sides of the bottle). Variations in the response of different lots of the substrate may cause variations in clotting time; therefore, measure the test samples and standards simultaneously on the same substrate. Average the clotting time, in s, of the duplicate samples, recording the time for the *Standard preparation* as T_S and that for the *Sample preparation* as T_U.

Calculation

Calculate the activity of the enzyme preparation by the equation

$$\text{Milk-clotting units/mL} = 100 \times (T_S/T_U) \times (D_S/D_U)$$

in which 100 is the activity assigned to the *Standard preparation*, D_S is the dilution factor for the *Standard preparation*, and D_U is the dilution factor for the *Sample preparation*.

[Note—The dilution factors should be expressed as fractions; for example, a dilution of 1 to 200 would be expressed as $^1/_{200}$.]

Change to read:

- **Pancreatin Activity**

Application and Principle: These procedures are used to determine the primary enzyme activities in pancreatin preparations.

Reference Standards

▲USP Bile Salts RS

Keep container tightly closed. Dry a portion at 105° for 4 h before using.

USP Pancreatin Amylase and Protease RS

Do not dry. Keep container tightly closed, and store in a freezer. Allow container to reach room temperature before opening.

USP Pancreatin Lipase RS

[**CAUTION—** Wash thoroughly after handling. Wear protective gloves. Avoid breathing dust.]. Store in a well-ventilated place. Keep container tightly closed. Store in a freezer.▲2S (FCC 12)

Amylase Activity

pH 6.8 phosphate buffer: On the day of use, dissolve 13.6 g of monobasic potassium phosphate in water to make 500 mL of solution. Dissolve 14.2 g of anhydrous dibasic sodium phosphate in water to make 500 mL of solution. Mix 51 mL of the monobasic potassium phosphate solution with 49 mL of the dibasic sodium phosphate solution. If necessary, adjust by the dropwise addition of the appropriate solution to a pH of 6.8.

Substrate solution: On the day of use, stir a portion of purified soluble starch equivalent to 2.0 g of dried substance with 10 mL of water, and add this mixture to 160 mL of water, add it to the hot solution, and heat to boiling, with continuous mixing. Cool to room temperature, and add water to make 200 mL.

Standard preparation: Weigh accurately about 20 mg of ▲*USP Pancreatin Amylase and Protease RS*▲2S (FCC 12) into a suitable mortar. Add about 30 mL of *pH 6.8 phosphate buffer*, and triturate for 5 to 10 min. Transfer the mixture with the aid of *pH 6.8 phosphate buffer* to a 50-mL volumetric flask, dilute with *pH 6.8 phosphate buffer* to volume, and mix. Calculate the activity, in USP Units of amylase activity per mL, of the resulting solution from the declared potency on the label of the Reference Standard.

Assay preparation: For Pancreatin having about the same amylase activity as the ▲*USP Pancreatin Amylase and Protease RS*,▲2S (FCC 12) weigh accurately about 40 mg of Pancreatin into a suitable mortar.

[NOTE—For Pancreatin having a different amylase activity, weigh accurately the amount necessary to obtain an *Assay preparation* having amylase activity per mL corresponding approximately to that of the *Standard preparation*.]

Add about 3 mL of *pH 6.8 phosphate buffer*, and triturate for 5 to 10 min. Transfer the mixture with the aid of *pH 6.8 phosphate buffer* to a 100-mL volumetric flask, dilute with *pH 6.8 phosphate buffer* to volume, and mix.

Procedure

Prepare four stoppered, 250-mL conical flasks, and mark them *S*, *U*, *BS*, and *BU*. Pipet into each flask 25 mL of *Substrate solution*, 10 mL of *pH 6.8 phosphate buffer*, and 1 mL of sodium chloride solution (11.7 in 1000), insert the stoppers, and mix. Place the flasks in a water bath maintained at 25° ± 0.1°, and allow them to equilibrate. To flasks *BU* and *BS* add 2 mL of 1 N hydrochloric acid, mix, and return the flasks to the water bath. To flasks *U* and *BU* add 1.0-mL portions of the *Assay preparation*, and to flasks *S* and *BS* add 1.0 mL of the *Standard preparation*. Mix each, and return the flasks to the water bath. After 10 min, accurately timed from the addition of the enzyme, add 2-mL portions of 1 N hydrochloric acid to flasks *S* and *U*, and mix. To each flask, with continuous stirring, add 10.0 mL of 0.1 N iodine VS, and immediately add 45 mL of 0.1 N sodium hydroxide. Place the flasks in the dark at a temperature between 15° and 25° for 15 min. To each flask add 4 mL of 2 N sulfuric acid, and titrate with 0.1 N sodium thiosulfate VS to the disappearance of the blue color. Calculate the amylase activity, in USP Units per mg, taken by the formula:

$$\text{Result} = 100(C_S/W_U)(V_{BU} - V_U)/(V_{BS} - V_S)$$

in which C_S is the amylase activity of the *Standard preparation*, in USP Units per mL; W_U is the amount, in mg, of Pancreatin taken; and V_U, V_S, V_{BU} and V_{BS} are the volumes, in mL, of 0.1 N sodium thiosulfate consumed in the titration of the solutions in flasks, *U*, *S*, *BU*, and *BS*, respectively.

Lipase Activity

Gum arabic solution: Centrifuge a 1:10 solution of gum arabic until clear. Use only the clear solution.

Olive oil substrate: Combine 165 mL of the *Gum arabic solution*, 20 mL of olive oil, and 15 g of crushed ice in the cup of an electric blender. Cool the mixture in an ice bath to 5°, and homogenize at high speed for 15 min, intermittently cooling in an ice bath to prevent the temperature from exceeding 30°. Test for suitability of mixing as follows: Place a drop of the homogenate on a microscope slide and gently press a cover slide in place to spread the liquid. Examine the entire field under high power (43 × magnification objective lens and 5 × magnification ocular), using an eyepiece equipped with a calibrated micrometer. The substrate is satisfactory if 90% of the particles do not exceed 2 µm in diameter and none exceeds 10 µm in diameter.

Buffer solution: Dissolve 60 mg of tris(hydroxymethyl)-aminomethane and 234 mg of sodium chloride in water to make 100 mL.

▲Bile salts solution: Prepare a solution containing 80.0 mg/mL of *USP Bile Salts RS*.▲2S (FCC 12)

Standard test dilution: Suspend about 200 mg of ▲*USP Pancreatin Lipase RS*,▲2S (FCC 12) accurately weighed, in about 3 mL of cold water in a mortar, triturate for 10 min, and add cold water to a volume necessary to produce a concentration of 8 to 16 USP Units of lipase activity per mL, based on the declared potency on the label of the Reference Standard. Maintain the suspension at 4°, and mix before using. For each determination, withdraw 5 to 10 mL of the cold suspension, and allow the temperature to rise to 20° before pipeting the exact volume.

Assay test dilution: Suspend about 200 mg of the Pancreatin sample, accurately weighed, in about 3 mL of cold water in a mortar, triturate for 10 min, and add cold water to a volume necessary to produce a concentration of 8 to 16 USP Units of lipase activity per mL, based on the estimated potency of the test material. Maintain the suspension at 4°, and mix before using. For each determination, withdraw 5 to 10 mL of the cold suspension, and allow the temperature to rise to 20° before pipeting the exact volume.

Procedure

Mix 10.0 mL of *Olive oil substrate*, 8.0 mL of *Buffer solution*, 2.0 mL of ▲*Bile salts solution*,▲2S (FCC 12) and 9.0 mL of water in a jacketed glass vessel of about 50-mL capacity, the outer chamber of which is connected to a thermostatically controlled water bath. Cover the mixture, and stir continuously with a mechanical stirring device.

With the mixture maintained at a temperature of 37° ± 0.1°, add 0.1 N sodium hydroxide, from a microburet inserted through an opening in the cover, to adjust potentiometrically the pH to 9.20, using a calomel-glass electrode system. Add 1.0 mL of *Assay test dilution*, and then continue adding the 0.1 N sodium hydroxide for 5 min to maintain the pH at 9.0. Determine the volume of 0.1 N sodium hydroxide added after each min.

In the same manner, titrate 1.0 mL of *Standard test dilution*.

Calculation

From the *Standard test dilution*, plot the volume of 0.1 N sodium hydroxide titrated against time. Using only the points that fall on the straight-line segment of the curve, calculate the

mean acidity released per min by the *Assay test dilution*. Taking into consideration dilution factors, calculate the lipase activity of the *Standard test dilution*, using the lipase activity of the ▲*USP Pancreatin Lipase RS*▲2S (FCC 12) stated on the label.

Protease Activity

Casein substrate: Place 1.25 g of finely powdered casein in a 100-mL conical flask containing 5 mL of water, shake to form a suspension, add 10 mL of 0.1 N sodium hydroxide, shake for 1 min, add 50 mL of water, and shake for about 1 h to dissolve the casein. Adjust the pH to about 8.0 ± 0.1, using 1 N sodium hydroxide or 1 N hydrochloric acid. Transfer the solution to a 100-mL volumetric flask, dilute with water to volume, and mix. Use this substrate on the day it is prepared.

Buffer solution: Dissolve 6.8 g of monobasic potassium phosphate and 1.8 g of sodium hydroxide in 950 mL of water in a 1000-mL volumetric flask, adjust to a pH of 7.5 ± 0.2, using 0.2 N sodium hydroxide, dilute with water to volume, and mix. Store this solution in a refrigerator.

Trichloroacetic acid solution: Dissolve 50 g of trichloroacetic acid in 1000 mL of water. Store this solution at room temperature.

Filter paper: Determine the suitability of the filter paper by filtering a 5-mL portion of *Trichloroacetic acid solution* through the paper and measuring the absorbance of the filtrate at 280 nm, using an unfiltered portion of the same *Trichloroacetic acid solution* as the blank. The absorbance is NMT 0.04. If the absorbance is more than 0.04, the filter paper may be washed repeatedly with *Trichloroacetic acid solution* until the absorbance of the filtrate, determined as above, is NMT 0.04.

Standard test dilution: Add about 100 mg of ▲*USP Pancreatin Amylase and Protease RS*,▲2S (FCC 12) accurately weighed, to 100.0 mL of *Buffer solution*, and mix by shaking intermittently at room temperature for about 25 min. Dilute quantitatively with *Buffer solution* to produce a concentration of about 2.5 USP Units of protease activity per mL, based on the potency declared on the label of the Reference Standard.

Assay test dilution: Add an amount of pancreatin sample equivalent to about 100 mg of ▲*USP Pancreatin Amylase and Protease RS*,▲2S (FCC 12) accurately weighed, to 100.0 mL of *Buffer Solution*, and mix by shaking intermittently at room temperature for 25 min. Dilute quantitatively with *Buffer solution* to obtain a dilution that corresponds in activity to the *Standard test dilution*.

Procedure

Label test tubes in duplicate S_1, S_2, and S_3 for the standard series, and U for the sample. Pipet into tubes S_1 2.0 mL, into S_2 and U 1.5 mL, and into S_3 1.0 mL of *Buffer solution*. Pipet into tubes S_1 1.0 mL, into S_2 1.5 mL, and into S_3 2.0 mL of the *Standard test silution*. Pipet into tubes U 1.5 mL of the *Assay Test dilution*. Pipet into one tube each of S_1, S_2, S_3, and U 5.0 mL of *Trichloroacetic acid solution*, and mix. Designate these tubes as S_{1B}, S_{2B}, S_{3B}, and U_B, respectively. Prepare a blank by mixing 3 mL of *Buffer solution* and 5 mL of *Trichloroacetic acid solution* in a separate test tube marked *B*. Place all the tubes in a 40° water bath, insert a glass stirring rod into each tube, and allow temperature equilibration. At zero time, add to each tube, at timed intervals, 2.0 mL of the *Casein substrate*, preheated to the bath temperature, and mix. Accurately timed, 60 min after the addition of the *Casein substrate*, stop the reaction in tubes S_1, S_2, S_3, and U by adding 5.0 mL of *Trichloroacetic acid solution* at the corresponding time intervals, stir, and remove all the tubes from the bath. Allow to stand for 10 min at room temperature to complete protein precipitation, and filter. The filtrates must be free from haze.

Determine the absorbances of each filtrate, in a 1-cm cell, at 280 nm, with a suitable spectrophotometer, using the intake from the blank (tube *B*) to set the instrument.

Calculation

Correct the absorbance values for the filtrates from tubes S_1, S_2, and S_3 by subtracting the absorbance values for the filtrates from tubes S_{1B}, S_{2B}, and S_{3B}, respectively, and plot the corrected absorbance values against the corresponding volumes of the *Standard test dilution* used. From the curve, using the corrected absorbance value ($U - U_B$ for the ▲Pancreatin▲2S (FCC 12) taken), and taking into consideration the dilution factors, calculate the protease activity, in USP Units, of the ▲Pancreatin▲2S (FCC 12) taken by comparison with that of the standard, using the protease activity stated on the label of ▲*USP Pancreatin Amylase and Protease RS*.▲2S (FCC 12)

- **Pepsin Activity**

Application: This procedure is used to determine the protease activity of pepsin preparations derived from porcine or other animal stomach tissue.

Reagents and Solutions

Dilute hydrochloric acid solution: Dilute 30 mL of 1.0 N hydrochloric acid with water to 1000 mL, then adjust to a pH of 1.6 ± 0.1 with 1.0 N hydrochloric acid, if necessary.

TCA solution: Prepare a 4.0% (w/v) solution of trichloroacetic acid in water.

Substrate solution: Weigh 5.0 g of *USP Hemoglobin Protease Substrate RS* into a large beaker. Add 100 mL of *Dilute hydrochloric acid solution* to the beaker, and stir using a magnetic stirrer. Once the hemoglobin is fully dissolved, adjust the pH of the solution to 1.6 ± 0.1 (use 1.0 N hydrochloric acid, dropwise, to adjust the pH as needed). Quantitatively transfer the solution to a 250-mL volumetric flask, then dilute with *Dilute hydrochloric acid solution* to volume. [Note—Prepare fresh daily.]

Standard solutions: Quantitatively prepare four serial dilutions of *USP Pepsin for Assay RS* in *Dilute hydrochloric acid solution* covering the activity range of 0.70–1.25 USP Pepsin U/mL. [Note—Prepare immediately before use.]

Sample solution: Quantitatively prepare a solution of pepsin in *Dilute hydrochloric acid solution* containing approximately 0.9–1.0 USP Pepsin U/mL. [Note—Prepare immediately before use.]

Procedure

For the standard curve, prepare test tubes as follows (use tubes that can accommodate and allow vortex mixing of a volume of NLT 16 mL). Separately transfer 1.0-mL aliquots of each of the *Standard solutions* into four test tubes—two tubes for the analysis of each *Standard Solution* and two blank tubes for each *Standard solution*. Prepare a substrate blank by transferring 1.0 mL of *Dilute hydrochloric acid solution* into a single test tube. A full set of tubes for creation of the standard curve will require seventeen test tubes.

For each pepsin sample being tested, prepare test tubes as follows. Separately transfer 1.0-mL aliquots of the *Sample Solution* into four test tubes—two tubes for the analysis of each sample and two blank tubes for each sample.

To each of the blank tubes (the substrate blank, the *Standard solution* blanks, and the *Sample solution* blanks), add 10.0 mL of *TCA Solution*, and mix by vortexing. Place all of the test tubes into a water bath maintained at 25° ± 0.1°. Add 5.0 mL of the *Substrate solution* (previously equilibrated to 25° ± 0.1°) to each of the blank tubes, and mix by vortexing. Using a stopwatch and starting at time equals zero, rapidly pipet 5.0 mL of the *Substrate solution* (previously equilbrated to 25° ± 0.1°) successively and at intervals of exactly 30 s into each of the tubes containing the *Sample solution* and *Standard solutions* for analysis. Vortex each tube immediately

after adding the *Substrate solution*, then return the tube to the water bath. Exactly 10.0 min after the addition of the *Substrate solution*, stop the reaction by rapidly pipetting 10.0 mL of the *TCA solution* into each analysis tube at intervals of exactly 30 s, immediately vortexing each tube after addition of the *TCA solution*. Remove all tubes from the water bath.

Allow all of the tubes (including blanks) to sit at room temperature for 25 min. Prepare clean test tubes for filtering the solutions, allowing two tubes for each solution to be filtered. Place funnels in each tube, and line the funnels with fluted or folded filter paper (use Whatman No. 41 ashless filter circles with a minimum diameter of 125 mm, or equivalent). After 25 min, vortex each tube, then transfer the contents of each tube into a clean test tube. Re-filter each of the filtrates through the same paper into a second clean test tube. Measure the absorbance of each of the filtrates at 280 nm using a suitable UV-visible spectrophotometer that has previously been zeroed with the substrate blank in a 1-cm quartz cell. Record the absorbance of each filtrate, and calculate the average absorbance reading for each *Standard solution*, *Sample solution*, and their respective blanks. Determine the net absorbance for each of the *Standard solutions* by subtracting the average absorbance of the *Standard solution* blank from the average absorbance of the corresponding *Standard solution*. Plot a standard curve of the net absorbance of each *Standard solution* versus its concentration, in mg/mL. Determine the slope (m) and y-intercept (b) of the resulting curve.

Calculation

One pepsin unit is defined as that quantity of enzyme that produces the equivalent of 1 µmol of tyrosine per min under the conditions of the assay.

Calculate the activity of the enzyme preparation by the equation:

$$\text{Pepsin Units/mg} = [(A_S - b) \times P]/(m \times C)$$

in which A_S is the average absorbance for the sample corrected for the average absorbance of the sample blanks; b is the y-intercept of the standard curve; P is the activity of the USP Pepsin for *Assay*RS used to prepare the *Standard solutions* (U/mg); m is the slope of the standard curve; and C is the concentration of the *Sample solution* (mg/mL).

- **Phospholipase Activity**

Application and Principle: This procedure is used to determine the phospholipase A_2 activity from extracts of porcine pancreatic tissue. The analysis is performed by potentiometric titration.

Apparatus

Automatic titrator: Use a suitable automatic recording titrator equipped with a stirred, thermostated, controlled-atmosphere titration cell (e.g., Radiometer Autotitrator).

Homogenizer: Use a suitable homogenizer (e.g., Biomixer; Fisher Scientific, Catalog No. 11-504-2-4, or equivalent).

Constant-temperature water bath: Set at 40° ± 0.1°.

Reagents and Solutions

Calcium chloride solution (0.3 M): Transfer 4.41 g of calcium chloride dihydrate to a 100-mL volumetric flask, dissolve in, and dilute to volume with water.

Sodium deoxycholate solution (0.016 M): Dissolve 0.67 g of sodium deoxycholate (Sigma Chemical Co., Catalog No. D6750) in 100 mL of water.

Sodium hydroxide solution (0.1 N): Use a standardized solution.

Substrate solution: Add the yolk of one fresh egg to 100 mL of deionized water and homogenize until a stable emulsion is obtained. Add 5 mL of the *Calcium Chloride Solution*, and mix.

Sample preparation: Dissolve an accurately weighed amount of enzyme preparation in 0.001 N hydrochloric acid, and dilute to obtain an enzyme activity of 10 to 80 units of activity per mL.

Procedure

Pre-equilibrate the *Substrate solution*, the *Sodium deoxycholate solution*, and about 50 mL of water to 40° in the water bath. Transfer 10 mL of the *Substrate solution* to the thermostated titration vessel. Add 5 mL of the *Sodium deoxycholate solution* and 10 mL of deionized water. *Blank*et the cell with nitrogen and equilibrate for approximately 5 min. Using the *Automatic titrator* filled with *0.1 N Sodium hydroxide solution*, adjust the pH of the solution to 8.0 ± 0.05. Monitor the consumption (if any) of sodium hydroxide for 5 min as a blank. Refill the *Automatic titrator*. Add 0.1 mL of *Sample solution* containing between 1 and 8 units of activity and start the *Automatic titrator*. Record the sodium hydroxide consumption for at least 5 min.

Calculation

One phospholipase unit is defined as the quantity of enzyme that produces 1 microequivalent of free fatty acid per min under the conditions of the test. Determine the rate, R, of titrant consumption during 0 to 3 min of the reaction.

[Note—The recorder trace must be linear during the first 3 min of the reaction.]

Determine the rate of titrant consumption (if any) during equilibration (blank) (R_B):

$$\text{Units/g} = (R \times N) - (R_B \times N)/W$$

in which R and R_B are the rates of titrant consumption of the sample and blank, respectively, in µL/min; N is the normality of the titrant; and W is the weight, in g, contained in 0.1 mL of the *Sample preparation* taken for the test.

- **Phytase Activity**

Application and Principle: This procedure is used to determine the activity of enzymes releasing phosphate from phytate. The assay is based on enzymatic hydrolysis of sodium phytate under controlled conditions by measurement of the amount of orthophosphate released.

Reagents and Solutions

[Note—All glassware must be acid washed, rinsed, and scrupulously cleaned to ensure the absence of phosphate.]

Acetate buffer (pH 5.5)**:** Dissolve 1.76 g of 100% acetic acid ($C_2H_4O_2$), 30.02 g of sodium acetate trihydrate ($C_2H_3O_2Na{\cdot}3H_2O$), and 0.147 g of calcium chloride dihydrate in about 900 mL of water. Transfer the solution into a 1000-mL volumetric flask, dilute to volume with water, and mix. The pH should be 5.50 ± 0.05.

Substrate solution: Dissolve 8.40 g of sodium phytate decahydrate ($C_6H_6O_{24}P_6Na_{12}{\cdot}10H_2O$) (MilliporeSigma) in 900 mL of *Acetate buffer*. Adjust the pH to 5.50 ± 0.05 at 37.0° ± 0.1° by adding 4 M acetic acid. Cool to ambient temperature. Quantitatively transfer the mixture to a 1000-mL volumetric flask, dilute to volume with *Acetate buffer*, and mix. Prepare fresh daily.

Nitric acid solution (27%): While stirring, slowly add 70 mL of 65% nitric acid to 130 mL of water.

Ammonium heptamolybdate solution: Dissolve 100 g of ammonium heptamolybdate tetrahydrate [$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$] in 900 mL of water in a 1000-mL volumetric flask. Add 10 mL of 25% ammonia solution, dilute to volume with water, and mix. This solution is stable for 4 weeks when stored at ambient temperature and shielded from light.

Ammonium vanadate solution: Dissolve 2.35 g of ammonium monovanadate (NH_4VO_3) in 400 mL of warm (60°) water. While stirring, slowly add 20 mL of *Nitric acid solution (27%)*. Cool to ambient temperature. Quantitatively transfer the mixture to a 1000-mL volumetric flask, dilute to volume with water, and mix. This solution is stable for 4 weeks when stored at ambient temperature and shielded from light.

Color/Stop solution: While stirring, add 250 mL of *Ammonium vanadate solution* to 250 mL of *Ammonium heptamolybdate solution*. Slowly add 165 mL of 65% nitric acid. Cool to ambient temperature. Quantitatively transfer the mixture to a 1000-mL volumetric flask, dilute to volume with water, and mix. Prepare fresh daily.

Potassium dihydrogen phosphate solution: Dry a sufficient amount of potassium dihydrogen phosphate (KH_2PO_4) in a vacuum oven at 100°–104° for 2 h. Cool to ambient temperature in a desiccator over dried silica gel.

In duplicate (solutions A and B), weigh approximately 0.245 g of dried potassium dihydrogen phosphate accurately to within 1 mg and dilute with *Acetate buffer* to 1 L to obtain solutions containing 1.80 mmol/L of potassium dihydrogen phosphate.

Phytase reference preparation (Highly concentrated phytase preparation)**:** This preparation can be obtained from Gist-Brocades, Delft, The Netherlands, with an assigned activity (by collaborative assay), or the activity of the reference preparation can be determined according to *Procedure 2*.

Phytase reference solutions, Procedure 1: Weigh an amount of *Phytase reference preparation* corresponding with 20,000 phytase units (FTU) accurately to within 1 mg in duplicate in 200-mL volumetric flasks. Dissolve in and dilute to volume with *Acetate buffer*, and mix. Dilute with *Acetate buffer* to obtain dilutions containing approximately 0.005, 0.01, 0.02, 0.03, and 0.04 FTU/mL.

Sample preparation, Procedure 1: Suspend or dissolve and dilute accurately weighed amounts of sample in *Acetate buffer* so that 2.0 mL of the final dilution (g/mL) will contain between 0.02 and 0.08 phytase units 0.01 and 0.04 FTU/mL.

Sample preparation, Procedure 1: Suspend or dissolve and dilute accurately weighed amounts of sample in *Acetate buffer* so that the final dilution (g/mL) will contain between 0.01 and 0.04 FTU/mL.

Sample preparation, Procedure 2: In duplicate, accurately weigh amounts of *Phytase reference preparation* and dissolve and dilute in *Acetate buffer* to obtain dilutions containing 0.06 ± 0.006 phytase units per 2.0 mL of the final dilution.

Procedures

Procedure 1: (Determination of the phytase activity) Transfer 2.00 mL of the *Sample preparation, Procedure 1*, and the *Phytase reference solutions, Procedure 1*, into separate 20- × 150-mm glass test tubes. Using a stopwatch and starting at time equals zero, in the order of the series and within regular time intervals, place the tubes into a 37.0° ± 0.1° water bath and allow their contents to equilibrate for 5 min. At time equals 5 min, in the same order of the series and with the same time intervals, add 4.0 mL of *Substrate solution* (previously equilibrated to 37.00 ± 0.10) to each test tube. Mix, and replace in the 37.0° ± 0.1° water bath. At time equals 65 min, in the same order and within the same time intervals, terminate the incubation by adding 4.0 mL of *Color/Stop solution*. Mix, and cool to ambient temperature. Prepare blanks by transferring 2.00 mL of the *Sample preparation, Procedure 1,* and the *Phytase reference solutions, Procedure 1,* into separate 20- × 150-mm glass test tubes. Using a stopwatch and starting at time equals zero, in the order of the series and within regular time intervals, place the tubes into a 37.0° ± 0.1° water bath and allow them to equilibrate for 5 min. At time equals 5 min, in the same order of the series and within the same time intervals, add 4.0 mL of *Color/Stop solution*. Mix, and cool to ambient temperature. Next add 4.00 mL of *Substrate solution* to the blank tubes, and mix.

Centrifuge all test tubes for 5 min at 3000 × g. Determine the absorbance of each solution at 415 nm in a 1-cm path-length cell with a suitable spectrophotometer, using water to zero the instrument.

Procedure 2: (Determination of the phytase activity of the *Phytase reference preparation*) Transfer 2.00 mL of *Sample preparation, Procedure 2,* and 2.00 mL (three times from *Potassium dihydrogen phosphate solution A* and two times from *B*) of *Potassium dihydrogen phosphate solutions* into separate 20- × 150-mm glass test tubes. Using a stopwatch and starting at time equals zero, in the order of the series and within regular time intervals, place the tubes into a 37.0° ± 0.1° water bath and allow their contents to equilibrate for 5 min. At time equals 5 min, in the same order of the series and within the same time intervals, add 4.0 mL of *Substrate solution* (previously equilibrated to 37.0° ± 0.1°) to the test tubes. Mix, and replace in the 37.0° ± 0.1° water bath. At time equals 35 min, in the same order and within the same time intervals, terminate the incubation by adding 4.0 mL of *Color/Stop solution*. Mix, and cool to ambient temperature.

Prepare blanks by transferring 2.00 mL of *Sample preparation, Procedure 2,* into separate 20- × 150-mm glass test tubes. Prepare *Reagent blanks* by transferring 2.00 mL of water into a series of five separate 20- × 150-mm glass test tubes. Add 4.0 mL of *Color/Stop solution* to all blank tubes and mix. Next add 4.0 mL of *Substrate solution*, and mix. Determine the absorbance of each solution at 415 nm in a 1-cm path-length cell with a suitable spectrophotometer, using water to zero the instrument.

Calculations

Calculation, Procedure 1: One phytase (fytase) unit (FTU) is the amount of enzyme that liberates inorganic phosphate at 1 µmol/min from sodium phytate 0.0051 mol/L at 37.00 at pH 5.50 under the conditions of the test. Calculate the corrected absorbance (sample minus blank) for each *Sample preparation* and *Phytase reference solution*. Plot the accurately calculated phytase activity (FTU/mL) of each *Phytase reference solution* against the corresponding absorbance. From the curve, determine the phytase activity in each *Sample preparation* (FTU/mL). Determine the phytase activity of the sample being tested:

$$\text{Activity (FTU/g)} = \text{phytase activity of the } \textit{Sample preparation}/\text{concentration of the } \textit{Sample preparation} \text{ (g/mL)}$$

Calculation, Procedure 2: Calculate the corrected absorbances A_R for each *Sample preparation* (absorbance *Phytase reference solution* minus corresponding absorbance blank) and for each *Potassium dihydrogen phosphate solution*, A_p (absorbance *Potassium dihydrogen phosphate solution* minus average absorbance reagent blank). Calculate *C*, the phosphate concentration of each *Potassium dihydrogen phosphate solution*:

$$(W \times 1000 \times 2)/\text{MW} = C \text{ (mmol/2 mL)}.$$

Calculate the absorbances *D* for each *Potassium dihydrogen phosphate solution* after correction for the amount of potassium dihydrogen phosphate weighed:

$$A_p/C = D \text{ (absorbance units/mmol of phosphate per 2 mL)}.$$

Calculate the average of results *D*, giving *E* (maximum allowable difference, 5%).

Calculate the activity for each *Phytase reference preparation*:

$$(A_R \times F)/(30 \times R \times E) = \text{FTU/g},$$

in which A_R equals the corrected absorbance of the *Phytase standard solution*; *F* equals the total dilution factor of the reference preparation; 30 equals the incubation time, in min; *R* equals sample weight, in g; *E* equals average of *D* factors; *W* equals the weight of potassium dihydrogen phosphate, in g; and MW equals the molecular weight of potassium dihydrogen phosphate, 136.09 (g/mol).

- **Plant Proteolytic Activity**

Application and Principle: This procedure is used to determine the proteolytic activity of papain, ficin, and bromelain. The assay is based on a 60-min proteolytic hydrolysis of a casein substrate at pH 6.0 and 40°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration; solubilized casein is then measured spectrophotometrically.

Reagents and Solutions

Sodium phosphate solution (0.05 M): Transfer 7.1 g of anhydrous dibasic sodium phosphate into a 1000-mL volumetric flask, dissolve in about 500 mL of water, dilute to volume with water, and mix. Add 1 drop of toluene as a preservative.

Citric scid solution (0.05 M): Transfer 10.5 g of citric acid monohydrate into a 1000-mL volumetric flask, dissolve in about 500 mL of water, dilute to volume with water, and mix. Add 1 drop of toluene as a preservative.

Phosphate–cysteine–EDTA buffer solution: Dissolve 7.1 g of anhydrous dibasic sodium phosphate in about 800 mL of water, and then dissolve in this solution 14.0 g of disodium EDTA dihydrate and 6.1 g of cysteine hydrochloride monohydrate.

Adjust to pH 6.0 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, then transfer into a 1000-mL volumetric flask, dilute to volume with water, and mix.

Trichloroacetic acid solution: Dissolve 30 g of trichloroacetic acid in 100 mL of water.

Casein substrate solution: Disperse 1 g (moisture-free basis) of Hammarsten-grade casein (United States Biochemical Corp., Catalog No. 12840, or equivalent) in 50 mL of *Sodium phosphate solution*, and heat for 30 min in a boiling water bath, with occasional agitation. Cool to room temperature, and with rapid and continuous agitation, adjust to pH 6.0 ± 0.1 by the addition of *Citric acid solution*.

[Note—Rapid and continuous agitation during the addition prevents casein precipitation.]

Quantitatively transfer the mixture into a 100-mL volumetric flask, dilute to volume with water, and mix.

Stock standard solution: Transfer 100.0 mg of USP Papain Reference Standard into a 100-mL volumetric flask, dissolve, and dilute to volume with *Phosphate–cysteine–EDTA buffer solution*, and mix.

Diluted standard solutions: Pipet 2, 3, 4, 5, 6, and 7 mL of *Stock standard solution* into a series of 100-mL volumetric flasks, dilute each to volume with *Phosphate–cysteine–EDTA buffer solution*, and mix by inversion.

Test solution: Prepare a solution from the enzyme preparation so that 2 mL of the final dilution will give a ΔA in the *Procedure* between 0.2 and 0.5. Weigh the sample accurately, transfer it quantitatively to a glass mortar, and triturate with *Phosphate–cysteine–EDTA buffer solution*. Transfer the mixture quantitatively into a volumetric flask of appropriate size, dilute to volume with *Phosphate–cysteine–EDTA buffer solution*, and mix.

Procedure

Pipet 5 mL of *Casein substrate solution* into each of a series of 25- × 150-mm test tubes, allowing three tubes for the enzyme unknown, six for a papain standard curve, and nine for enzyme blanks. Equilibrate the tubes for 15 min in a water bath maintained at 40° ± 0.1°. Starting the stopwatch at zero time, rapidly pipet 2 mL of each of the *Diluted standard solutions*, and 2-mL portions of the *Test solution*, into the equilibrated substrate. Mix each by swirling, stopper, and place the tubes back in the water bath. After 60.0 min, add 3 mL of *Trichloroacetic acid solution* to each tube. Immediately mix each tube by swirling.

Prepare enzyme blanks containing 5.0 mL of *Casein substrate solution*, 3.0 mL of *Trichloroacetic acid solution*, and 2.0 mL of one of the appropriate *Diluted standard solutions* or the *Test solution*.

Return all tubes to the water bath, and heat for 30.0 min, allowing the precipitated protein to coagulate completely. Filter each mixture through Whatman No. 42, or equivalent, filter paper, discarding the first 3 mL of filtrate. The subsequent filtrate must be perfectly clear. Determine the absorbance of each filtrate in a 1-cm cell at 280 nm, with a suitable spectrophotometer, against its respective blank.

Calculation

One papain unit (PU) is defined in this assay as that quantity of enzyme that liberates the equivalent of 1 µg of tyrosine per h under the conditions of the assay.

Prepare a standard curve by plotting the absorbances of filtrates from the *Diluted standard solutions* against the corresponding enzyme concentrations, in mg/mL. By interpolation from the standard curve, obtain the equivalent concentration of the filtrate from the *Test solution*.

Calculate the activity of the enzyme preparation taken for analysis as follows:

$$PU/mg = (A \times C \times 10)/W$$

in which A is the activity of USP Papain Reference Standard, in PU per mg; C is the concentration, in mg/mL, of Reference Standard from the standard curve, equivalent to the enzyme unknown; 10 is the total volume, in mL, of the final incubation mixture; and W is the weight, in mg, of original enzyme preparation in the 2-mL aliquot of *Test solution* added to the incubation mixture.

- **Proteolytic Activity Bacterial (PC)**

Application and Principle: This procedure is used to determine protease activity, expressed as PC units, of preparations derived from *Bacillus subtilis* var. and *Bacillus licheniformis* var., and it may be used to determine the activity of other proteases at pH 7.0. The assay is based on a 30-min proteolytic hydrolysis of casein at 37° and pH 7.0. Unhydrolyzed casein is removed by filtration, and the solubilized casein is determined spectrophotometrically.

Reagents and Solutions

Casein: Use Hammarsten-grade casein (United States Biochemical Corp., Catalog No. 12840, or equivalent).

Tris buffer (pH 7.0): Dissolve 12.1 g of enzyme-grade (or equivalent) tris(hydroxymethyl)aminomethane in 800 mL of water, and titrate with 1 N hydrochloric acid to pH 7.0. Transfer into a 1000-mL volumetric flask, dilute with water to volume, and mix.

TCA solution: Dissolve 18 g of trichloroacetic acid and 19 g of sodium acetate trihydrate in 800 mL of water in a 1000-mL volumetric flask, add 20 mL of glacial acetic acid, dilute with water to volume, and mix.

Substrate solution: Dissolve 6.05 g of enzyme-grade tris(hydroxymethyl)aminomethane in 500 mL of water, add 8 mL of 1 N hydrochloric acid, and mix. Dissolve 7 g of *Casein* in this solution, and heat for 30 min in a boiling water bath, stirring occasionally.

Cool to room temperature, and adjust to pH 7.0 with 0.2 N hydrochloric acid, adding the acid slowly, with vigorous stirring, to prevent precipitation of the casein. Transfer the mixture into a 1000-mL volumetric flask, dilute with water to volume, and mix.

Sample preparation: Using *Tris buffer*, prepare a solution of the sample enzyme preparation so that 2 mL of the final dilution will contain between 10 and 44 bacterial protease units.

Procedure

Pipet 10.0 mL of the *Substrate solution* into each of a series of 25- × 150-mm test tubes, allowing one tube for each enzyme test, one tube for each enzyme blank, and one tube for a substrate blank. Equilibrate the tubes for 15 min in a water bath maintained at 37° ± 0.1°.

Starting the stopwatch at zero time, rapidly pipet 2.0 mL of the *Sample preparation* into the equilibrated substrate. Mix, and replace the tubes in the water bath. Add 2 mL of *Tris buffer* (instead of the *Sample preparation*) to the substrate blank. After exactly 30 min, add 10 mL of *TCA solution* to each enzyme incubation and to the substrate blank to stop the reaction. Heat the tubes in the water bath for an additional 30 min to allow the protein to coagulate completely.

At the end of the second heating period, shake each tube vigorously, and filter through 11-cm Whatman No. 42, or equivalent, filter paper, discarding the first 3 mL of filtrate.

[Note—The filtrate must be perfectly clear.]

Determine the absorbance of each sample filtrate in a 1-cm cell, at 275 nm, with a suitable spectrophotometer, using the filtrate from the substrate blank to set the instrument at zero. Correct each reading by subtracting the appropriate enzyme blank reading, and record the value so obtained as A_U.

Standard curve

Transfer 100.0 mg of L-tyrosine, chromatographic-grade or equivalent (Aldrich Chemical Co.), previously dried to constant weight, to a 1000-mL volumetric flask. Dissolve in 60 mL of 0.1 N hydrochloric acid. When completely dissolved, dilute the solution with water to volume, and mix thoroughly. This solution contains 100 µg of tyrosine in 1.0 mL. Prepare three more dilutions from this stock solution to contain 75.0, 50.0, and 25.0 µg of tyrosine per mL. Determine the absorbance of the four solutions at 275 nm in a 1-cm cell on a suitable spectrophotometer versus 0.006 N hydrochloric acid. Prepare a plot of absorbance versus tyrosine concentration.

Calculation

One bacterial protease unit (PC) is defined as that quantity of enzyme that produces the equivalent of 1.5 µg/mL of L-tyrosine per min under the conditions of the assay.

From the *Standard curve*, and by interpolation, determine the absorbance of a solution having a tyrosine concentration of 60 µg/mL. A figure close to 0.0115 should be obtained. Divide the interpolated value by 40 to obtain the absorbance equivalent to that of a solution having a tyrosine concentration of 1.5 µg/mL, and record the value thus derived as A_S.

Calculate the activity of the sample enzyme preparation by the equation:

$$PC/g = (A_U/A_S) \times (22/30W)$$

in which 22 is the final volume, in mL, of the reaction mixture; 30 is the time, in min, of the reaction; and W is the weight, in g, of the sample in 2 mL of the *Sample preparation*.

- **Proteolytic Activity, Fungal (HUT)**

Application and Principle: This procedure is used to determine the proteolytic activity, expressed as hemoglobin units on the tyrosine basis (HUT), of preparations derived from *Aspergillus oryzae* var. and *Aspergillus niger* var., and it may be used to determine the activity of other proteases at pH 4.7. The test is based on the 30-min enzymatic hydrolysis of a hemoglobin substrate at pH 4.7 and 40°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized hemoglobin in the filtrate is determined spectrophotometrically.

Reagents and Solutions

Hemoglobin: Use Hemoglobin substrate powder (Sigma Chemical Co., Catalog No. H2625) or a similar high-grade material that is completely soluble in water.

Acetate buffer solution: Dissolve 136 g of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) in sufficient water to make 500 mL. Mix 25.0 mL of this solution with 50.0 mL of 1 M acetic acid, dilute to 1000 mL with water, and mix. The pH of this solution should be 4.7 ± 0.02.

Substrate solution: Transfer 4.0 g of the *Hemoglobin* into a 250-mL beaker, add 100 mL of water, and stir for 10 min to dissolve. Immerse the electrodes of a pH meter in the solution, and while stirring continuously, adjust the pH to 1.7 by adding 0.3 N hydrochloric acid. After 10 min, adjust the pH to 4.7 by adding 0.5 M sodium acetate. Transfer the solution into a 200-mL volumetric flask, dilute to volume with water, and mix. This solution is stable for about 5 days when refrigerated.

Trichloroacetic acid solution: Dissolve 140 g of trichloroacetic acid in about 750 mL of water. Transfer the solution to a 1000-mL volumetric flask, dilute to volume with water, and mix thoroughly.

Sample preparation: Dissolve an amount of the sample in the *Acetate buffer solution* to produce a solution containing, in each mL, between 9 and 22 HUT. (Such a concentration will produce an absorbance reading, in the procedure below, within the preferred range of 0.2 to 0.5.)

Procedure:

Pipet 10.0 mL of the *Substrate solution* into each of a series of 25- × 150-mm test tubes: one for each enzyme test and one for the substrate blank. Heat the tubes in a water bath at 40° for about 5 min. To each tube, except the substrate blank, add 2.0 mL of the *Sample preparation*, and begin timing the reaction at the moment the solution is added; add 2.0 mL of the *Acetate buffer solution* to the substrate blank tube. Close the tubes with No. 4 rubber stoppers, and tap each tube gently for 30 s against the palm of the hand to mix. Heat each tube in a water bath at 40° for exactly 30 min, and then rapidly pipet 10.0 mL of the *Trichloroacetic acid solution* into each tube. Shake each tube vigorously against the stopper for about 40 s, and then allow to cool to room temperature for 1 h, shaking each tube against the stopper at 10- to 12-min intervals during this period. Prepare enzyme blanks as follows: Heat, in separate tubes, 10.0 mL of the *Substrate solution* and about 5 mL of the *Sample preparation* in the water bath for 30 min, then add 10.0 mL of the *Trichloroacetic acid solution* to the *Substrate solution*, shake well for 40 s, and to this mixture add 2.0 mL of the preheated *Sample preparation*. Shake again, and cool at room temperature for 1 h, shaking at 10- to 12-min intervals.

At the end of 1 h, shake each tube vigorously, and filter through 11-cm Whatman No. 42, or equivalent, filter paper, refiltering the first half of the filtrate through the same paper. Determine the absorbance of each filtrate in a 1-cm cell, at 275 nm, with a suitable spectrophotometer, using the filtrate from the substrate blank to set the instrument to zero. Correct each reading by subtracting the appropriate enzyme blank reading, and record the value so obtained as A_U.

[Note—If a corrected absorbance reading between 0.2 and 0.5 is not obtained, repeat the test using more or less of the enzyme preparation as necessary.]

Standard curve:

Transfer 100.0 mg of L-tyrosine, chromatographic-grade, or equivalent (Aldrich Chemical Co.), previously dried to constant weight, to a 1000-mL volumetric flask. Dissolve in 60 mL of 0.1 N hydrochloric acid. When the L-tyrosene is completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 100 µg of tyrosine in 1.0 mL. Prepare three more dilutions from this stock solution to contain 75.0, 50.0, and 25.0 µg of tyrosine per mL. Determine the absorbance of the four solutions at 275 nm in a 1-cm cell on a suitable spectrophotometer versus 0.006 *N* hydrochloric acid. Prepare a plot of absorbance versus tyrosine concentration. Determine the slope of the curve in terms of absorbance per µg of tyrosine. Multiply this value by 1.10, and record it as A_S. A value of approximately 0.0084 should be obtained.

Calculation

One HUT unit of proteolytic (protease) activity is defined as that amount of enzyme that produces, in 1 min under the specified conditions, a hydrolysate whose absorbance at 275 nm is the same as that of a solution containing 1.10 µg/mL of tyrosine in 0.006 N hydrochloric acid.

Calculate the HUT per g of the original enzyme preparation by the equation:

$$\text{HUT/g} = (A_U/A_S) \times (22/30W)$$

in which 22 is the final volume of the test solution; 30 is the reaction time, in min; and W is the weight, in g, of the original sample taken.

[NOTE—The value for A_S, under carefully controlled and standardized conditions, is 0.0084; this value may be used for routine work in lieu of the value obtained from the standard curve, but the exact value calculated from the standard curve should be used for more accurate results and in cases of doubt.]

- **PROTEOLYTIC ACTIVITY, FUNGAL (SAP)**

Application and Principle: This procedure is used to determine proteolytic activity, expressed in spectrophotometric acid protease units (SAP), of preparations derived from *Aspergillus niger* var. and *Aspergillus oryzae* var., and it may be used to determine the activity of other proteases at pH 3.0. The test is based on a 30-min enzymatic hydrolysis of a Hammarsten Casein Substrate at pH 3.0 and at 37°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized casein in the filtrate is determined spectrophotometrically.

Reagents and Solutions

Casein: Use Hammarsten-grade casein (United States Biochemical Corp., Catalog No. 12840, or equivalent).

Glycine–hydrochloric acid buffer (0.05 M): Dissolve 3.75 g of glycine in about 800 mL of water. Add 1 N hydrochloric acid until the solution is pH 3.0, determined with a pH meter. Quantitatively transfer the solution to a 1000-mL volumetric flask, dilute with water to volume, and mix.

TCA solution: Dissolve 18.0 g of trichloroacetic acid and 11.45 g of anhydrous sodium acetate in about 800 mL of water, and add 21.0 mL of glacial acetic acid. Quantitatively transfer the solution to a 1000-mL volumetric flask, dilute with water to volume, and mix.

Substrate solution: Pipet 8 mL of 1 N hydrochloric acid into about 500 mL of water, and with continuous agitation, disperse 7.0 g (moisture-free basis) of *Casein* into this solution. Heat for 30 min in a boiling water bath, stirring occasionally, and cool to room temperature. Dissolve 3.75 g of glycine in the solution, and using a pH meter, adjust to pH 3.0 with 0.1 N hydrochloric acid. Quantitatively transfer the solution to a 1000-mL volumetric flask, dilute with water to volume, and mix.

Sample preparation: Using *Glycine–hydrochloric acid buffer*, prepare a solution of the sample enzyme preparation so that 2 mL of the final dilution will give a corrected absorbance of enzyme incubation filtrate at 275 nm (ΔA, as defined in the *Procedure*) between 0.200 and 0.500. Weigh the enzyme preparation, quantitatively transfer it to a glass mortar, and triturate with *Glycine–Hydrochloric acid buffer*. Quantitatively transfer the mixture to an appropriately sized volumetric flask, dilute with *Glycine–hydrochloric acid buffer* to volume, and mix.

Procedure

Pipet 10.0 mL of the *Substrate solution* into each of a series of 25- × 150-mm test tubes, allowing at least two tubes for each sample, one for each enzyme blank, and one for a substrate blank. Stopper the tubes, and equilibrate them for 15 min in a water bath maintained at 37° ± 0.1°. At zero time, start the stopwatch, and rapidly pipet 2.0 mL of the *Sample preparation* into the equilibrated substrate. Mix by swirling, and replace the tubes in the water bath.

[NOTE—Keep the tubes stoppered during incubation.]

Add 2 mL of *Glycine–hydrochloric acid buffer* (instead of the *Sample preparation*) to the substrate blank. After exactly 30 min, add 10 mL of *TCA solution* to each enzyme incubation and to the substrate blank to stop the reaction. In the following order, prepare an enzyme blank containing 10 mL of *Substrate solution*, 10 mL of *TCA Solution*, and 2 mL of the *Sample preparation*. Heat all tubes in the water bath for 30 min, allowing the precipitated protein to coagulate completely. At the end of the second heating period, cool the tubes in an ice bath for 5 min, and filter through Whatman No. 42 filter paper, or equivalent. The filtrates must be perfectly clear. Determine the

absorbance of each filtrate in a 1-cm cell at 275 nm with a suitable spectrophotometer, against the substrate blank. Correct each absorbance by subtracting the absorbance of the respective enzyme blank.

Standard curve

Transfer 181.2 mg of L-tyrosine, chromatographic-grade or equivalent (Sigma Chemical Co.), previously dried to constant weight, to a 1000-mL volumetric flask. Dissolve in 60 mL of 0.1 N hydrochloric acid. When the L-tyrosine is completely dissolved, dilute the solution with water to volume, and mix thoroughly. This solution contains 1.00 µmol of tyrosine per 1.0 mL. Prepare dilutions from this stock solution to contain 0.10, 0.20, 0.30, 0.40, and 0.50 µmol/mL. Determine against a water blank the absorbance of each dilution in a 1-cm cell at 275 nm. Prepare a plot of absorbance versus µmol of tyrosine per mL. A straight line must be obtained. Determine the slope and intercept for use in the *Calculation* below. A value close to 1.38 should be obtained. The slope and intercept may be calculated by the least squares method as follows:

$$\text{Slope} = [n\Sigma\,(MA) - \Sigma\,(M)\,\Sigma(A)]/[n\Sigma\,(M^2) - (\Sigma M)^2]$$

$$\text{Intercept} = [\Sigma(A)\,\Sigma(M^2) - \Sigma(M)\,\Sigma(MA)]/[n\Sigma\,(M^2) - (\Sigma M)^2],$$

in which n is the number of points on the standard curve, M is the µmol of tyrosine per mL for each point on the standard curve, and A is the absorbance of the sample.

Calculation

One spectrophotometric acid protease unit is that activity that will liberate 1 µmol of tyrosine per min under the conditions specified. The activity is expressed as follows:

$$\text{SAP/g} = (\Delta A - I) \times 22/(S \times 30 \times W)$$

in which ΔA is the corrected absorbance of the enzyme incubation filtrate; I is the intercept of the *Standard curve*; 22 is the final volume of the incubation mixture, in mL; S is the slope of the *Standard curve*; 30 is the incubation time, in min; and W is the weight, in g, of the enzyme sample contained in the 2.0-mL aliquot of *Sample preparation* added to the incubation mixture in the *Procedure*.

- **Pullulanase Activity**

Application and Principle: This procedure is used to determine the pullulanase activity of pullulanase preparations and pullulanase preparations blended with glucoamylase. Acarbose is used in this method to inhibit the glucoamylase enzyme. The method is based on measuring the increase in reducing sugars formed by a 30-min hydrolysis of pullulan at 40° and pH 5.0. The increase in reducing sugars is measured spectrophotometrically at 520 nm using a modified Nelson-Somogyi procedure.

Reagents and Solutions

Citrate buffer (with 0.1 g/L acarbose, pH 5.0): Dissolve 10.5 g of citric acid monohydrate in 950 mL of water, adjust the pH to 5.0 ± 0.005 using 5 N sodium hydroxide, and dilute to 1000 mL. Dissolve 0.1 g of acarbose (Bayer HealthCare) into 1000 mL of *Citrate buffer* at pH 5.0.

Nelson's color reagent: Dissolve 25.0 g of ammonium molybdate tetrahydrate in 300 mL of water, and carefully add 20.0 mL of concentrated sulfuric acid while stirring. Dissolve 3.0 g of sodium arsenate heptahydrate in 25 mL of water. Slowly add this solution to the ammonium molybdate solution with stirring. Dilute this solution to 500 mL with water.

Somogyi's copper reagent: Dissolve 14.0 g of anhydrous dibasic sodium phosphate and 20.0 g of potassium sodium tartrate tetrahydrate into 250 mL of water. Add 60.0 g of 1 M sodium hydroxide solution. Dissolve 4.0 g of cupric sulfate pentahydrate into 25 mL of water. Add this solution to the tartrate solution. Add 90.0 g of anhydrous sodium sulfate while stirring. Dilute the final solution to 500 mL.

Glucose standards: Dissolve 800 mg of previously dried anhydrous D-glucose in 100 mL of the *Citrate buffer.* Prepare glucose standards containing 80, 120, 160, and 250 µg/mL of glucose.

Pullulan substrate: Dissolve 150 mg of pullulan (Sigma Chemical Co, Catalog No. P-4516, or equivalent) in 49.80 g of the *Citrate buffer.* Prepare daily.

Sample preparation: Dissolve an accurately weighed amount of the enzyme preparation in *Citrate buffer* and dilute in *Citrate buffer* to obtain an enzyme activity of 0.015 to 0.040 activity units per mL.

Procedure

Transfer 1.0 mL aliquots of *Pullulan substrate* to separate 15- × 150-mm test tubes and equilibrate for 15 min in a 40° ± 0.1° water bath. At time equals zero and at 30 s intervals, add 1.0 mL of the respective samples, and mix. Exactly 30.0 min after addition of the samples, add 2.0 mL of *Somogyi's copper reagent* to each tube to terminate the reaction. Mix thoroughly, and allow the tubes to come to room temperature.

Run sample blanks by adding 2.0 mL of *Somogyi's copper reagent* to 1.0 mL of sample, then add 1.0 mL of substrate. The sample blanks should not be incubated at 40°.

Prepare a standard curve by adding 2.0 mL of each *Glucose standard* and 2.0 mL of *Somogyi's copper reagent* to a test tube, and mix. Run a buffer blank to subtract from the *Glucose standards* by adding 2.0 mL of buffer and 2.0 mL *Somogyi's copper reagent* to a test tube, and mix. The *Glucose standards* should not be incubated at 40°. Loosely stopper samples, blanks, and *Glucose standards* containing *Somogyi's copper reagent*, and place them in a vigorously boiling water bath for exactly 25.0 min. Cool in an ice bath for 5 min. Add 2.0 mL of *Nelson's color reagent* to each tube, and mix thoroughly to dissolve any red precipitate that might be present. Let the solutions stand for 5 min. Add 2.0 mL of water to each tube, and mix. Measure the absorbance of all solutions at 520 nm, using water as the reference. Mix the contents of each tube before transferring them to the cuvette.

Calculations

One pullulanase unit (PUN) is the amount of activity that, under the conditions of the test, will liberate reducing sugars equivalent to 1 µmol of glucose per min. Determine the linear regression line for absorbance versus 2 times the glucose concentration (µg/mL) in the standards. Use the slope, a, in the following equation to determine the activity in the enzyme preparation:

$$\text{PUN/g} = (A_S - A_B) / (a \times W \times 180 \times 30)$$

in which A_S is the absorbance of the sample; A_B is the absorbance of the blank; W is the weight, in g, of the enzyme preparation contained in the 1.0 mL of *Sample preparation* taken for analysis; 180 is the molecular weight of glucose; and 30 is the incubation time in min.

- **Transglutaminase Activity (Glutaminyl-peptide γ-Glutaminyltransferase)**

Application and Principle: This procedure is used to determine transglutaminase activity in preparations derived from *Streptoverticillium mobaraense* var. The assay is based on the enzymatic formation of a glutamic acid γ-hydroxamate in a glutaminyl residue in the substrate peptide with another substrate, hydroxylamine. The amount of the glutamic acid γ-hydroxamate formed as a red complex with ferric ion in acidic conditions at 37° is measured spectrophotometrically.

Reagents and Solutions

Substrate solution: Transfer 12.110 g of Tris[tri(hydroxymethyl)aminomethane], 3.475 g of hydroxylamine hydrochloride, 1.624 g of glutathione, and 5.060 g of carbobenzyloxy-glutaminylglycine into a 500-mL beaker. Add 350 mL of water, and using a magnetic stirrer, mix well. Adjust the pH to 6.0 with appropriate concentrations (usually 1 N and 6 N) of hydrochloric

acid. Quantitatively transfer this mixture into a 500-mL volumetric flask, and dilute to volume with water.

Stopping solution: Prepare a 3 N hydrochloric acid solution by diluting concentrated hydrochloric acid (ca. 36%) four-fold with water. Make a 12% trichloroacetic acid (TCA) solution by transferring 12.0 g of TCA into a 100-mL volumetric flask, adding water to dissolve the TCA, and diluting to volume with water. Prepare a 5% solution of ferric chloride ($FeCl_3$) in 0.1 N hydrochloric acid by transferring 5.0 g of ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) into a 100-mL volumetric flask, adding 0.1 N hydrochloric acid to dissolve the ferric chloride, and diluting to volume with 0.1 N hydrochloric acid. On the day of use, combine all three solutions (3 N hydrochloric acid, 12% TCA, and 5% ferric chloride) in equal volumes in a beaker, and using a magnetic stirrer, mix well.

0.2 M Tris-HCl buffer (pH 6.0): Transfer 18.11 g of Tris[tri(hydroxymethyl)aminomethane] into a 500-mL beaker. Add 350 mL of water, and using a magnetic stirrer, mix well. Adjust the pH to 6.0 with appropriate concentrations (usually 1 N and 6 N) of hydrochloric acid. Quantitatively transfer this mixture into a 500-mL volumetric flask, and dilute to volume with water.

Sample solution: Place 100 mg of sample, accurately weighed, into a 100-mL beaker, and add about 45 mL of *0.2 M Tris-HCl buffer*. Using a magnetic stirrer, mix well at room temperature for 30 min. Quantitatively transfer the mixture into a 50-mL volumetric flask, and dilute to volume with *0.2 M Tris-HCl buffer*.

Procedure

Calibration curve: Transfer 64.8 mg of L-glutamic acid γ-monohydroxamate standard, accurately weighed, into a suitable flask, and add 10 mL of *0.2 M Tris-HCl buffer*. Dilute this solution sequentially in five steps each by a geometric factor of 2 with *0.2 M Tris-HCl buffer*. Transfer 200 µL of each dilution by pipet into individual test tubes, and incubate at 37° for 1 min. Add 2 mL of *Substrate solution*, previously incubated at 37° for 10 min, to each tube, and mix vigorously with a vortex mixer. Further incubate the mixtures for exactly 10 min, add 2 mL of *Stopping solution* to each tube, and start a stopwatch. Mix vigorously with the vortex mixture, and separate any insoluble material by centrifugation at 1500 × g for 10 min at about 25°. Measure the absorbance of the supernatant in each tube at 525 nm exactly 30 min after the addition of the *Stopping solution*. Plot the absorbance against the amount of L-glutamic acid γ-monohydroxamate, and obtain a standard calibration curve used to calculate the amount of glutamic acid γ-monohydroxamate in carbobenzyloxy-glutaminylglycine from the absorbance obtained in the analysis of the samples.

Analysis of Samples

Transfer 200 µL of *Sample solution* by pipet into a test tube, and incubate at 37° for 1 min. Add 2 mL of *Substrate solution*, previously incubated at 37° for 10 min, and mix vigorously using a vortex mixer. Further incubate the mixture for exactly 10 min, add 2 mL of *Stopping solution*, and start a stopwatch. Mix vigorously using a vortex mixer, and separate any insoluble material by centrifugation at 1500 × g for 10 min at about 25°. Measure the absorbance of the supernatant at 525 nm exactly 30 min after adding the *Stopping solution*.

For the blank, place 200 µL of *Sample solution* into a test tube, and incubate at 37° for 1 min. Add 2 mL of *Stopping solution*, and mix vigorously using a vortex mixer. Further incubate for exactly 10 min, and add 2 mL of *Substrate solution*, previously incubated at 37° for 10 min, and start a stopwatch. Mix vigorously using a vortex mixer. Separate any insoluble material by centrifugation at 1500 × g for 10 min. Measure the absorbance of the supernatant at 525 nm exactly 30 min after adding the *Substrate solution*.

Calculation

One unit of enzyme activity is defined as the amount of enzyme that catalyzes the transglutamination of 1 µmol of substrate into product in 1 min under the conditions of the assay. The specific activity of Transglutaminase is defined as:

$$\text{Transglutaminase activity (U/g)} = C \times T_S \times 1/S \times {}^{1}/_{10},$$

in which C is the concentration, in micromoles per milliliter, of hydroxamate in the *Sample solution* (obtained from the standard calibration curve); T_S is the total volume, in milliliters, of *Sample solution*; S is the mass, in grams, of the sample taken; and 10 is the reaction time in min.

Transglutaminase

Transfer of acyl groups between the γ-carboxyamide group of peptide-bound glutamine residues and various amines, including the ε-amino group of peptide-bound lysine, to form intra- and intermolecular ε-(γ-glutamyl)lysine crosslinks (see *Table 3*).

Table 3

Trivial Name	Classification	Source	Systematic Name (IUB)	IUB No.
Transglutaminase	Acyltransferase or aminotransferase	*Streptoverticillium mobaraense* var.	R-glutaminyl-peptide: amine γ-glutamyltransferase	2.3.2.13

- **Trypsin Activity**

Application and Principle: This procedure is used to determine the trypsin activity of trypsin preparations derived from purified extracts of porcine or bovine pancreas.

Reagents and Solutions

Fifteenth molar phosphate buffer (pH 7.6): Dissolve 4.54 g of monobasic potassium phosphate in sufficient water to make 500 mL of solution. Dissolve 4.73 g of anhydrous dibasic sodium phosphate in sufficient water to make 500 mL of solution.

Mix 13 mL of the monobasic potassium phosphate solution with 87 mL of the anhydrous dibasic sodium phosphate solution.

Substrate solution: Dissolve 85.7 mg of *N*-benzoyl-l-arginine ethyl ester hydrochloride, suitable for use in assaying trypsin, in sufficient water to make 100 mL.

[Note—Determine the suitability of the substrate and check the adjustment of the spectrophotometer by performing the assay using USP Trypsin Reference Standard.]

Dilute 10.0 mL of this solution to 100.0 mL with *Fifteenth molar phosphate buffer*. Determine the absorbance of this solution at 253 nm in a 1-cm cell, with a suitable spectrophotometer, using water as the blank and maintaining the cell temperature at 25° ± 0.1°. Adjust the absorbance of the solution, if necessary, by the addition of *Fifteenth molar phosphate buffer* so that it measures NLT 0.575 and NMT 0.585. Use this solution within a period of 2 h.

Sample Preparations: Dissolve a sufficient amount of sample, accurately weighed, in 0.001 N hydrochloric acid to produce a solution containing about 3000 USP trypsin units in each mL. Prepare three dilutions using 0.001 N hydrochloric acid so that the final solutions will contain 12, 18, and 24 USP trypsin units in each 0.2 mL. Use these concentrations in the *Procedure* below.

Procedure

Conduct the test in a spectrophotometer equipped to maintain a temperature of 25° ± 0.1° in the cell compartment.

Determine the temperature in the reaction cell before and after the measurement of absorbance to ensure that the temperature does not change by more than 0.5°.

Pipet 0.2 mL of 0.001 N hydrochloric acid and 3.0 mL of *Substrate solution* into a 1-cm cell. Place this cell in the spectrophotometer, and adjust the instrument so that the absorbance will read 0.050 at 253 nm. Pipet 0.2 mL of the *Sample preparation* containing 12 USP units into another 1-cm cell. Add 3.0 mL of *Substrate solution*, and place the cell in the spectrophotometer. At the same time the *Substrate solution* is added, start a stopwatch, and read the absorbance at 30-s intervals for 5 min. Repeat the procedure with the *Sample preparations* containing 18 and 24 USP units. Plot curves of absorbance versus time for each concentration, and use only those values that form a straight line to determine the activity of the trypsin. Discard the values on the plateau, and take the average of the results from the three concentration levels as the actual activity of the trypsin.

Calculations

One USP trypsin unit is the activity causing a change in the absorbance of 0.003/min under the conditions specified in this assay.

Calculate the number of USP trypsin units per mg at each level by the equation:

$$\text{USP trypsin units} = (A_1 - A_2)/(T \times W \times 0.003)$$

in which A_1 is the absorbance straight-line final reading; A_2 is the absorbance straight-line initial reading; T is the elapsed time, in min, between the initial and final readings; and W is the weight, in mg, of trypsin in the volume of solution used in determining the absorbance.

[1] Shugar, D. 1952. The measurement of lysozyme activity and the ultraviolet inactivation of lysozyme. *Biochimica et Biophysica Acta*. 8:302–309.

Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
APPENDIX V -- ENZYME ASSAYS	Tongtong Xu Associate Scientific Liaison +1 (301) 692-3659 Ext.3659	FI2020 Food Ingredients

Page Information

- FCC 12 2S - page 1871
- FCC 12 - page 1366
- FCC 9 - page 413

Viebrock, Lauren

From: 岡戸信夫 <nokado@snc-enzymes.co.jp>
Sent: Friday, July 02, 2021 4:09 AM
To: Viebrock, Lauren
Subject: [EXTERNAL] Re: GRN 000965
Attachments: GRN 965-Response to FDA Questions_22 June'21.pdf

Follow Up Flag: Follow up
Flag Status: Flagged

Dear Loren,

Thank you for your email and your comments.
Please refer to the attached for our answer in detail to your inquiry regarding confidentiality.

Yours sincerely,

++
Nobuo Okado
Quality Assurance
Shin Nihon Chemical Co., Ltd.
19-10, Showa-cho, Anjo, Aichi 446-0063, Japan
Tel: +81-566-75-5555 Fax: +81-566-75-0010
Mail; nokado@snc-enzymes.co.jp
++

2021年6月23日(水) 0:40 Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>:

> Dear Mr. Okado,
>
> During our review of GRAS Notice No. 000965, we noted that GRN 000965 contained references that were designated confidential. Data and information used to support a GRAS conclusion must be generally available and generally recognized and therefore cannot be confidential. Please clarify whether that information is considered confidential or if it was incorrectly designated confidential. If it is considered confidential, please provide a brief narrative to explain how experts could get to a GRAS conclusion of safety without the confidential information. Please let me know if you have any questions.
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> We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,

Lauren

Lauren VieBrock
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

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Responses to the U.S. FDA's Questions Related to GRAS Notice No. GRN 000965

In response to the United States Food and Drug Administration's question (dated 22 June 2021) pertaining to GRAS Notice No. GRN 000965, as submitted by Shin Nihon for arabinase enzyme preparation produced by *Aspergillus tubingensis*, below please find the additionally requested information.

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Kasamoto S (2014a) [unpublished]. *Bacterial Reverse Mutation Test with Sumizyme AG (Experiment No. F072 [365-184]).* (Final Report). [*Confidential*]. Prepared by Public Interest Incorporated Foundation, Biosafety Research Center (BSRC) for Shin Nihon Chemical Co., Ltd.

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Although the above cited references should not have been designated as "Confidential", it should also be emphasized that consistent with the general recognition requirement of GRAS determinations that the data and information relied on to support the safety and GRAS status of the arabinase enzyme preparation for its intended use must be generally available and accepted by qualified experts (*e.g.,* through publication in the scientific literature), the results of the above-listed studies also were published in Okado *et al*. (2019). General recognition of arabinase's GRAS conclusion was supported by the unanimous consensus rendered by the GRAS Panel after reviewing the study reports, as well as the results of these studies as published in Okado *et al*. (2019). Although the GRAS Panel that evaluated the safety of the enzyme preparation was provided the Okado *et al*. (2019) publication, along with the unpublished study reports (as reflected in the GRAS Panel Report), the unpublished study reports do not provide any additional pivotal information upon which the determination of GRAS was made that would not have also been discussed in the Okado *et al*. (2019) publication. As such, access solely to the published data (*i.e*., the Okado *et al.* 2019 publication), in the absence of the unpublished study reports, would not preclude other experts from reaching the same conclusions as the GRAS Panel, regarding the GRAS status of the enzyme for its intended use. Consistent with this, only the Okado *et al*. (2019) publication is referenced in the GRAS Notice in the context of the above listed study reports. The information provided in the Okado *et al*. (2019) publication, along with other publicly available data, as discussed in the GRAS Panel Report and GRAS Notice, provided the basis for the GRAS Panel to conclude regarding the safety and GRAS status of the enzyme preparation for its intended use.

Overall, while we confirm that the study reports were erroneously designated as "Confidential", the results of the studies were published in Okado *et al*. (2019) and as such the fact that the reports are unpublished does not in our opinion have any implications related to the common knowledge element (*i.e.*, general recognition) of the GRAS standard and the ability of other experts to reach the same conclusions as the GRAS Panel that evaluated the safety of the arabinase enzyme preparation. We hope that this answers the question related to the designation of the unpublished references as "Confidential"; however, please let us know should you require any further information regarding the above.

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Lauren

Lauren VieBrock
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
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