JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Thermal Stability of α-Amylase from *Aspergillus oryzae* Entrapped in Polyacrylamide Gel

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To determine the suitability as a time−temperature indicator for dielectric pasteurization processes, the thermal stability (50−75 °C) of *Aspergillus oryzae* α-amylase immobilized in polyacrylamide gel in phosphate buffer, mashed potatoes, and minced shrimp was examined. Changing the cross-linking agent concentration from 3.3 to 5.3% and adding 2% salt did not markedly affect the thermal stability of the immobilized α-amylase. Thermal inactivation was first order, and immobilization generally improved the thermal stability of α-amylase. *z* values of the immobilized system in test food systems were 10.2 °C (phosphate buffer), 8.45 °C (minced shrimp), and 7.78 °C (mashed potatoes).

KEYWORDS: α-Amylase; inactivation kinetics; polyacrylamide gel; time−temperature indicator; pasteurization

INTRODUCTION

Microbial testing is a reference method for monitoring process lethality with microbes having *z* values from 5 to 12 °C useful for validating pasteurization processes (*1*). Microbial testing is often a time-consuming, burdensome, and costly proposition for food processors (*2*, *3*). This has prompted the development of enzyme-based time−temperature indicators (TTIs) to monitor process lethality. Ideally, the thermal stability of a TTI should be somewhat higher than that of the target pathogen under the same experimental conditions; in this way residual enzyme activity can be detected following heat treatment. α-Amylases (α-1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) are of particular interest because these enzymes are inexpensive and commercially available, and the assay is fast, simple, and inexpensive. Inactivation of free α-amylase may is generally first order (*1*, *4*−*6*).

End-point assays of endogenous enzyme activity have been used to determine the adequacy of a cooking process but are poor candidates for the quantitative monitoring of thermal inactivation (*7*−*14*). Use of exogenous enzyme-based TTIs shows more promise, but there has been little research conducted in this area, particularly in food systems. Inactivation of horseradish peroxidase (*15*−*17*) or α-amylase (*4*, *5*) immobilized on glass beads has been studied, but not in foods. Entrapped *B. amyloliquefaciens* α-amylase in silicone particles or stainless steel capsules (*1*) has been studied in limited food applications. For TTIs in pasteurization processes (*18*), specifically those involving microwave or radio frequency (RF) heating, a TTI with dielectric properties that match the food would be important so that uniform coupling of microwave or RF radiation can be assured.

A TTI based upon *Aspergillus oryzae* α-amylase immobilized in polyacrylamide gel provides a number of advantages over current designs, including ease of preparation, physical durability, easy separation of the gel from foods, a fast, simple, and inexpensive assay, and the simplicity of adjusting the sensitivity of enzyme assay.

The objectives of this study were (1) to determine the temperature optima and thermal stability of free and immobilized α-amylase entrapped in polyacrylamide gel and (2) to determine inactivation kinetic parameters for free and immobilized α-amylase in buffer solution and two food systems, minced shrimp and mashed potatoes.

MATERIALS AND METHODS

Enzyme Assay. *A. oryzae* α-amylase (EC 3.2.1.1) (Sigma Aldrich Co., St. Louis, MO) with a specific activity of 39 units/mg of solid or 185 units/mg of protein (by biuret method) was used.

The 5 DE maltrodextrin substrate [Maltrin M040, Grain Processing Corp. (Muscatine, IA)] was prepared as described by Strumeyer (*19*). To block reducing ends, 5 g of maltodextrin was dispersed into 20 mL of distilled water and heated for 20−30 s until completely dissolved. The suspension was then diluted to 90 mL with distilled water and cooled in an ice bath. Then 10 mL of cold sodium borohydride (0.15 g of $NaBH_4$/10 mL) was added and stirred using a magnetic stirrer for 2 min. The solution was stored under refrigeration overnight before use and is stable for several days.

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10.1021/jf020906j CCC: $25.00
Published on Web 08/05/2003

Immediately prior to assay, 0.4 mL of acetone was added to 20 mL of the $NaHB_4$-treated Maltrin and allowed to incubate for 20 min at room temperature to remove any unreacted borohydride. This substrate solution was adjusted to pH 7.0 with 1 M acetic acid, and then the volume was adjusted to 100 mL with 0.05 M phosphate buffer, pH 6.9.

This maltodextrin substrate (1.0 mL) was added to 1.0 mL of α-amylase solution preequilibrated to 30 °C. One unit of α-amylase will liberate 1.0 mg of maltose from a 5 DE maltodextrin solution per minute at pH 6.9 and 30 °C. Reaction was terminated by adding 1.0 mL of 0.01 M 3,5-dinitrosalicylic acid solution (DNS) (5.0 g of 3,5-dinitrosalicylic acid in 20 mL of 2 N NaOH, 30 g of sodium tartrate diluted to 100m with distilled, deionized water). Color was developed by placing the reaction mixture in a boiling water bath for exactly 5 min, cooling on ice, and diluting to 10 mL with distilled water prior to spectrophotometric measurement. The activity of α-amylase (milligrams of maltose equivalent per minute) (30 °C) was measured at A_{540nm} (UV−vis spectrophotometer, model 400, Spectral Instruments, Tucson, AZ).

Enzyme Immobilization. *α-Amylase Solution.* For free α-amylase measurements, 2 units/mL of α-amylase was prepared in 25 mL of cold 0.05 M phosphate buffer, pH 7.1. The solution was kept on ice and used within 4 h. For immobilization, the concentration of α-amylase solution was calculated such that 1−2 units per piece (ca. 0.5 × 0.5 × 0.5 cm) of polyacrylamide gel (4.35 mg/mL) were recovered following immobilization.

Immobilization Procedure. Two polyacrylamide gel systems were employed. One system contained 66.6% (v/v) of solution 1 [29.0 g of a 20% (w/v) solution of acrylamide with 1.0 g of a 3.3% (w/v) solution of *N*,*N*′-methylenebisacrylamide], 31.3% (v/v) of α-amylase solution, 2.1% (v/v) of ammonium persulfate solution, and 30 μL of *N*,*N*,*N*′,*N*′-tetramethyethylenediamine (TEMED).

A second polyacrylamide gel system contained 66.6% (v/v) of solution 2 [28.5 g of a 20% (w/v) solution of acrylamide with 1.5 g of a 5.3% (w/v) solution of *N*,*N*′-methylenebisacrylamide]. Others gel constituents were as listed. These concentrations of bisacrylamide were selected because the resulting gels were easy to incorporate into test foods and were neither too soft nor too brittle to handle.

The immobilized α-amylase-containing gel was prepared by blending the α-amylase solution into either solution 1 or solution 2 with stirring on a magnetic stir plate with a Teflon-coated stirring bar for 2 min at low speed. Ammonium persulfate was added and stirred for 1 min, followed by TEMED, stirred for 0.5 min. The mixture was allowed to polymerize between two glass plates separated with flexible rubber tubing around three sides (0.5 cm spacing) for 4 h at 4 °C. Gel was stored at 4 °C in Ziploc plastic bags until used. Two percent NaCl gels were prepared by adding reagent grade NaCl to gels immediately after the α-amylase solution had been added. For routine experiments, the gel was cut into 0.5 × 0.5 × 0.5 cm pieces with a single-edge razor blade.

Assay of Immobilized α-Amylase. Three pieces of gel (∼0.36 g) were dispersed in 3.0 mL of cold extraction buffer (0.05 M phosphate buffer, pH 7.1) on ice using a hand-held tissue grinder. The dispersion was incubated at 4−6 °C for 30 min and centrifuged in a 1.7 mL microcentrifuge tube (5415 Eppendorf centrifuge, Brinkmann Instrument, Inc., New York) for 4 min. The liquid fraction was assayed as described for free α-amylase.

Temperature Optima Experiments. For free α-amylase, α-amylase [0.3 mL (2 units)] was added to a glass test tube containing 0.7 mL of 0.05 M phosphate buffer, pH 7.1, and 1.0 mL of maltodextrin solution equilibrated at a preset temperature (30−75 °C) in a water bath (Lab Line Instruments, Inc., Melrose Park, IL). The test tube was covered with aluminum foil. After the desired heating time (3−20 min), 1 mL of DNS solution was immediately added to the sample and enzyme activity determined. Experiments were conducted in duplicate for each temperature.

Recovered α-Amylase from the Gel. To determine whether immobilization affected temperature optima, α-amylase was extracted from polyacrylamide gel (5.3% cross-linking agent, 2% NaCl). One milliliter of maltodextrin solution and 0.7 mL of 0.05 M phosphate buffer, pH 7.1, was equilibrated to a preset temperature (50−75 °C), and then 0.3 mL of the liquid fraction from crushed immobilized α-amylase gel was added. After the desired heating time (4−20 min), the liquid fraction was recovered, and 1 mL of DNS solution was immediately added to the mixture to stop α-amylase reaction; activity was determined as previously described. Experiments were conducted in duplicate for each temperature.

Immobilized α-Amylase. Two units of α-amylase immobilized in one piece (0.5 × 0.5 × 0.5 cm) of polyacrylamide gel (5.3% bisacrylamide with 2% NaCl) were weighed, cut into four pieces (0.25 × 0.50 × 0.25 cm), and then quickly placed into the glass test tube containing 1.0 mL of maltodextrin solution and 1.0 mL of 0.05 M phosphate buffer, pH 7.1, with 1% NaCl, equilibrated to a preset temperature (50−70 °C) in a water bath. The test tubes were covered with aluminum foil and held for the desired treatment time (4−20 min). The activity was determined as previously described. Experiments were conducted in duplicate for each temperature.

Thermal Stability. *Free α-Amylase.* α-Amylase (0.5 mL, 20 units) was added to a series of glass tubes containing 4.5 mL of 0.05 M phosphate buffer, pH 7.1, previously equilibrated at temperatures between 50 and 75 °C, covered with aluminum foil, and incubated for 3 min. Five milliliters of ice-cold phosphate buffer was immediately added to the sample to halt further inactivation. The tubes were quickly transferred to an ice bath and held no longer than 1.5 h before assay. Assays were conducted as previously described. Experiments were conducted in duplicate for each temperature.

Immobilized α-Amylase. Three pieces [(0.5 × 0.5 × 0.5 cm), 2 units/piece (5.3% bisacrylamide with 2% NaCl] of known weight were placed into a covered glass test tube containing 1.5 mL of 0.05 M phosphate buffer, pH 7.1, with 1% NaCl, equilibrated to a preset temperature (50−75 °C) in a water bath as previously described, and incubated for 3 min. Ice-cold buffer (1.5 mL) was immediately added to the α-amylase−buffer solution to halt further inactivation. Assays were as described above.

Thermal Inactivation Experiments. *Free α-Amylase in Buffer Solution.* Phosphate buffer (3.8 mL of 0.05 M, pH 7.1) was preheated to the desired temperature in a water bath as previously described. α-Amylase solution (4 mL or 80 units) was added, and at predetermined time intervals, 0.1 mL (2 units) of the heated α-amylase solution was transferred to a glass tube containing 0.9 mL of cold phosphate buffer held on an ice bath (*16*) and then assayed within 1.5 h. Three experiments were conducted in duplicate for each temperature.

Immobilized α-Amylase in Buffer Solution. Three pieces of immobilized enzyme gel were weighed and transferred to a glass test tube containing 1 mL of 0.05 M phosphate buffer, pH 7.1, previously heated to the desired treatment temperature (55−70 °C). After the desired heating time (0.5−30 min), 2 mL of ice-cold buffer was immediately added to stop inactivation. The glass tubes were quickly transferred to the ice bath (*16*) and assayed within 1.5 h for residual α-amylase activity. Three experiments were conducted in duplicate for each temperature.

Immobilized α-Amylase Inactivation in Minced Shrimp. Frozen shrimp (*Penaeus monodon*) 90/100 shrimp/kg, headed with intact exoskeleton in 0.5 kg frozen blocks, were obtained from a local retailer and stored at −35 °C. Shrimp were placed in Ziploc plastic bags and thawed under running water at 20 °C, peeled, deveined, and macerated in a blender for 1 min at room temperature. One gram of ground shrimp was placed into 1.5 × 1.5 cm polyethylene pouches. Three pieces of gel (0.5 × 0.5 × 0.5 cm) containing 1−2 units of α-amylase (5.3% bisacrylamide with 2% NaCl) were weighed and placed into the geometric center of the ground shrimp sample. Excess air was removed from the pouch before heat-sealing (Impulse heat sealer, model MP8, Midwest Pacific, Grainger Inc., Bellevue, WA). A thermocouple was used to measure the temperature at the geometric center of each pouch (model 600-1020, Barnant Inc., Barrington, IL). The gels remained in the shrimp matrix no longer than 2 h prior to heat treatment. The pouches were submerged in a water bath at 55, 60, 65, or 70 °C. The time−temperature profiles were recorded. Pouches were removed at designated intervals (0.5−90 min), cooled in ice water (2 °C), and kept cold and assayed within 2 h. Experiments were conducted in duplicate for each temperature.

Inactivation of Immobilized α-Amylase in Mashed Potatoes. Mashed potatoes were prepared by mixing potato flakes (Safeway Inc., Oakland,

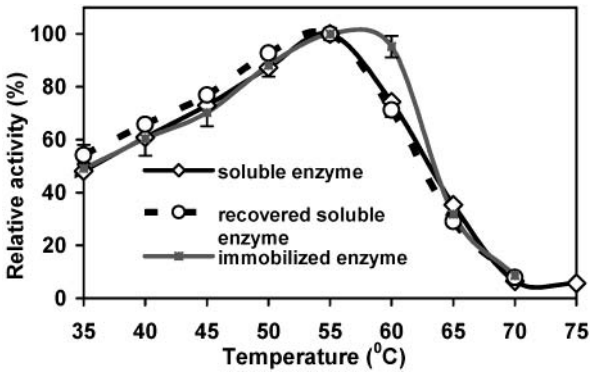

Figure 1. Temperature and activity of α-amylase in 0.05 M phosphate buffer, pH 7.1.

CA), distilled water, and reagent grade NaCl [20:6:3.9 (w/w)]. One gram of mashed potatoes was used for each treatment following the experimental procedure described above for ground shrimp.

Correction of *D* and *z* Values. Time−temperature profiles for the shrimp and mashed potato heat treatments were used to calculate an appropriate come-up period for kinetic models. The thermal lag during cooling was neglected, as it was very short. t_c was calculated using the method for correcting the *D* value of microorganisms (*20*)

$$t_c = \int_{t_{45}}^{t} 10^{(T-T_h)}/Z \, dt$$

where t was the time of the applied heat treatment, t_{45} was the time needed for the sample to reach 45 °C, the temperature at which *Listeria* destruction begins (*21*); T was the temperature of the sample during heating at time t; T_h was the heating temperature under study; and Z was the preliminary value obtained without correction.

The corrected *D* and *z* values were recalculated using the corrected thermal times. The procedure was repeated until the relative error for *z* values between two successive calculations was within 5% (*22*, *23*).

RESULTS AND DISCUSSION

Temperature Optima. The similar shapes of the α-amylase activity profiles for the free and recovered soluble enzyme following immobilization were similar over the entire range of temperatures tested (**Figure 1**), indicating that the immobilization process had a minor affect on temperature optima and suggesting that, under these experimental conditions, the immobilization protocol may provide a microenvironment similar to that of the bulk solution. The enzyme may have a high inherent stability independent of stabilizing α-amylase−matrix interactions (*4*). The optimum temperature of 55 °C for free α-amylase confirms that of prior studies (*24*).

The temperature optima of entrapped α-amylase shifted toward a higher temperature (55−60 °C) (**Figure 1**). Kennedy (*25*) suggests that an increase in temperature optima could result from a lower temperature in the gel microenvironment compared to the bulk solution.

Temperature Stability. Increased thermal stability for immobilized α-amylase from *A. oryzae* (*4*) and *B. licheniformis* (*5*) immobilized on glass beads has been reported. In this study, we observed that the immobilized α-amylase (with 5.3% bisacrylamide) was less stable than the free enzyme between 55 and 70 °C (**Figure 2**). This may have resulted from changes in the microenvironment of the gel matrix as the temperature increased, inducing conformational changes in the enzyme that enhanced its susceptibility to thermal inactivation. A more likely explanation is that a stabilized hydrogen-bonded structure formed between amide groups in acrylamide and α-amylase may have been affected as temperature increased.

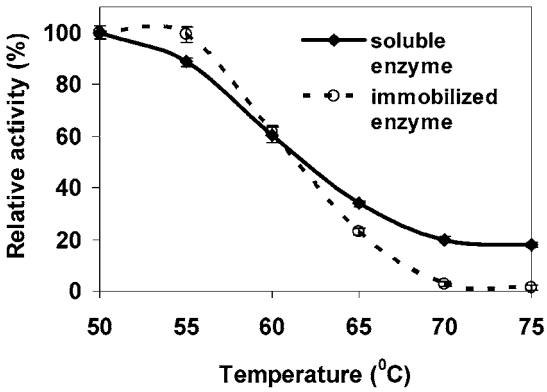

Figure 2. Thermostability of α-amylase in 0.05 M phosphate buffer, pH 7.1, after 3 min of heating.

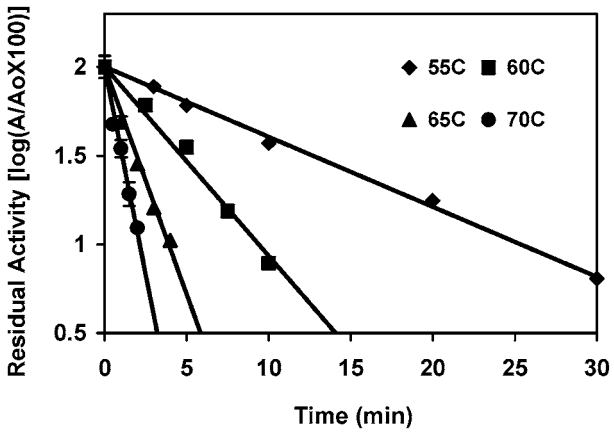

Figure 3. Inactivation of free α-amylase in 0.05 M phosphate buffer, pH 7.1, at 55–70 °C.

Kinetic Experiments. *Free and Immobilized α-Amylases in Buffer Solution.* The thermal inactivation of free and immobilized α-amylase (3.3 and 5.3% bisacrylamide) is first order (**Figure 3**). The *k* and *D* values for the free α- amylase (**Table 1**) indicate, in general, greater denaturation compared with the immobilized counterparts. The E_a value of the free α-amylase was 156 kJ/mol, with values between 200 and 275 kJ/mol for the immobilized enzyme in buffer and food systems. α-Amylase stability was higher at lower concentrations of bisacrylamide (3.3%) (**Table 1**).

At temperatures ≥60 °C, the immobilized α-amylase was relatively less stable than the free α-amylase. A larger difference in stability between lower and higher test temperatures indicates a TTI system with greater temperature sensitivity (**Table 1**). Despite differences in stability, changing the concentration of cross-linking agent did not have a marked effect upon the kinetic of inactivation properties of the α-amylase.

Kinetic Data Analysis. The thermal inactivations for free and immobilized α-amylase were first order in buffer and food systems (**Figure 4**). The following equations can be used to model α-amylase inactivation (*21*, *22*):

$$dA/dt = -kA \quad (1)$$

The integrated form of eq 1 is

$$A - A_0 = -kt^z/z \quad (2)$$

where A represents the residual α-amylase activity (mg of maltose equivalent/min), A_0 represents the initial α-amylase activity (mg of maltose equivalent/min), and t and k represent

Table 1. Kinetic Parameters of Free and Immobilized α-Amylase (IE)[a]

α-amylase/food system	temp (°C)	*D* value (min)	*k* (min^{-1})	*z* value (°C)	E_a (kJ/mol)
free/buffer	55–70			13.9	156
	55	25.3	0.0910		
	60	9.39	0.245		
	65	3.90	0.591		
	70	2.14	1.08		
IE_1/buffer	55–70			10.9	198
	55	63.7	0.036		
	60	16.0	0.144		
	65	6.45	0.357		
	70	2.56	0.900		
IE_2/buffer	55–70			10.2	212
	55	58.8	0.039		
	60	9.22	0.250		
	65	4.22	0.546		
	70	1.79	1.29		
IE_2/minced shrimp	55–70			8.32	256
	55	270	0.009		
	60	59.9	0.039		
	65	12.0	0.192		
	70	4.56	0.505		
IE_2/mashed potatoes	55–70			7.78	275
	55	303	0.008		
	60	78.7	0.029		
	65	7.28	0.316		
	70	4.82	0.478		

[a] IE_1 = immobilized α-amylase with 3.3% bisacrylamide. IE_2 = immobilized α-amylase with 5.3% bisacrylamide. *D* and *z* values were calculated from **Figures 3–8**. *k* values were calculated from *D* values, and E_a values were obtained from the regression slope of the logarithms of *k* values against 1/*T*.

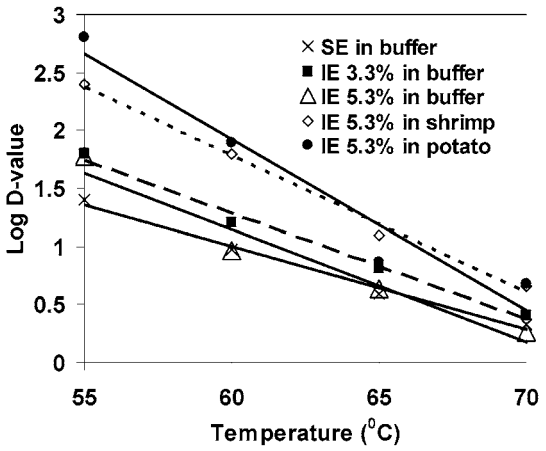

Figure 4. Thermal inactivation curves for soluble α-amylase (SE) in 0.05 M phosphate buffer, pH 7.1, or immobilized α-amylase (IE) in 3.3 or 5.3% bisacrylamide in 0.05 M phosphate, pH 7.1, minced shrimp, or mashed potatoes.

time (min) and reaction rate constant (min^{-1}) at a particular temperature, respectively.

Reaction rate (*k*) increases with temperature, normally following an Arrhenius relationship

$$k = k_0 e^{-Ea/RT} \quad (3)$$

where k_0 is the rate constant of the TTI at a reference temperature (T_0), E_a is the activation energy, *T* is absolute temperature (273.1 + °C), and *R* is the universal gas constant (8.314 J/mol·K or 1.987 cal/mol·K). Alternatively, *k* can be calculated from *D* values (*4*). The *D* value is the heating time

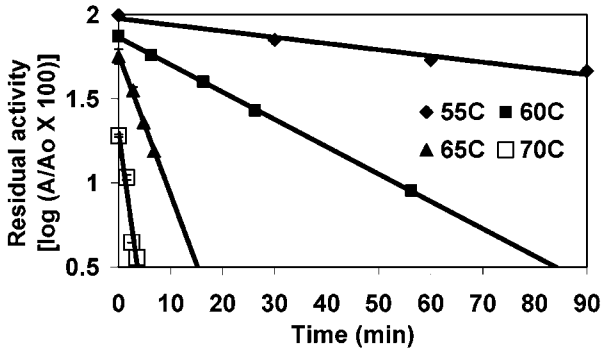

Figure 5. Thermal inactivation of immobilized α-amylase (5.3% bisacrylamide, 2% NaCl) in minced shrimp.

required to inactivate 90% of the α-amylase, measured as a decrease from initial activity, at a given temperature. The *D* value is defined as the negative reciprocal slope of the regression $\log(A/A_0)$ versus *t*.

$$k = 2.303/D \quad (4)$$

The *z* value is defined as the change in temperature required to yield a 10-fold change in *D* value and was calculated by determining the negative reciprocal slope from semilogarithmic plots of *D* as a function of temperature (°C) (**Figure 4**; **Table 1**). Values of E_a (activation energy) were obtained from the regression slope of the logarithms of *k* values against 1/*T*. E_a can be calculated from *z* values

$$E_a = 2.303 T_{min} T_{max}/z \quad (5)$$

where T_{min} and T_{max} are minimum and maximum temperature (K), respectively, at a given temperature range (*22*).

First-order inactivation kinetics has been observed by others for free *A. oryzae* α-amylase in sodium acetate buffer (*4*), for *B. licheniformis* α-amylase in Tris-HCl buffer above 85 °C (6) but not at lower temperatures (*5*), and for *B. amyloliquefaciens* α-amylase in tris(hydroxymethyl)aminomethane buffer (*1*). Horseradish peroxidase in phosphate buffer exhibited biphasic behavior with an inactivation order of ~1.7 (*16*). For immobilized enzyme, *B. amyloliquefaciens* α-amylase sealed in stainless steel capsules exhibited first-order inactivation (*1*). However, when *A. oryzae* (*4*) and *B. licheniformis* α-amylases (*5*) were immobilized on glass beads, biphasic behavior was observed. Even though these discrepancies may result from the different experimental conditions used, it clearly indicates that thermal stability experiments must be conducted for each newly developed immobilized enzyme system for which thermal inactivation kinetics would be important.

Thermal Inactivation of Immobilized α-Amylase in Minced Shrimp and Mashed Potatoes. The thermal stability of immobilized α-amylase was affected by the food matrix used (**Figures 5** and **6**; **Table 1**). The *z* values for minced shrimp and mashed potatoes were similar (**Table 1**) despite the widely differing chemical compositions of these foods. Immobilized α-amylase was more stable in minced shrimp and mashed potatoes than in phosphate buffer. Thermal stability of the immobilized α-amylase was greater in mashed potatoes over the entire range of temperatures studied except at 65 °C, where stability was greater in minced shrimp. α-Amylase may be more stable in the presence of substrates such as potato starch. Potato contains relatively high amounts of low molecular weight carbohydrates, and diffusion of these moieties into the gel during inactivation experiments may have improved α-amylase stabil-

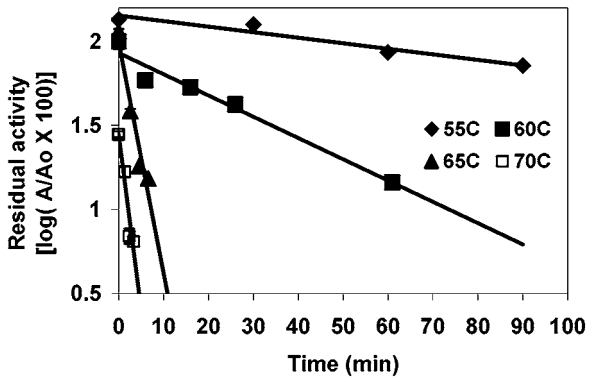

Figure 6. Thermal inactivation of immobilized α-amylase (5.3% bisacrylamide, 2% NaCl) in minced mashed potatoes.

ity. Covalently bound carbohydrate has been shown to stabilize hydrolase enzymes (*25*, *26*). Determining the degree of this effect requires further study.

These results suggest that the TTI developed here may have wide applicability for validating pasteurization processes for low-acid foods (up to 70 °C), particularly for pasteurization processes employing microwave and RF heating sources when matching the dielectric properties of the food with the marker would be an important criterion. However, because different food matrices may interact differently with the TTI, matrices should be individually tested if optimal temperature sensitivity and accuracy are required. This TTI is sufficiently stable with *z* values of 7−10 °C slightly above that of bacterial food pathogens and suitable for validating food pasturization processes.

ACKNOWLEDGMENT

We give special thanks to Hao Feng, University of Illinois, for his assistance with this research project.

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Received for review August 23, 2002. Revised manuscript received May 19, 2003. Accepted June 5, 2003. This research was supported by the USDA International Marketing Program for Agricultural Commodities and Trade (IMPACT), USDA National Research Initiative Competitive Grants Program (NRI CGP 2002-35201-11683) Chiangmai University, the Kingdom of Thailand, and Washington State University.

JF020906J