substances suggest the potential for the development of plant cell reactors for the continuous production of such metabolites.

ACKNOWLEDGMENT

This work was partly supported by the National Sea Grant College Program under Grant No. NA83AA-D-00017, Project No. A/I-18. An award from the National Food Brokers Association to S.M.M. is also acknowledged.

Registry No. Protease, 9001-92-7.

LITERATURE CITED

Apte, P. V.; Kaklij, G. S.; Heble, M. R. *Plant Sci. Lett.* **1979**, *14*, 57.
Biesboer, D. D. *Plant Cell Rep.* **1983**, *2*, 137.
Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
Brockbank, W. J.; Lynn, K. R. *Biochim. Biophys. Acta* **1979**, *578*, 13.
Brodelius, P.; Deus, B.; Mosbach, K.; Zenk, M. H. *Fed. Eur. Biochem. Soc. Lett.* **1979**, *103*, 93.
Carpenter, D. C.; Lovelace, F. E. *J. Chem. Soc.* **1943**, *65*, 2364.
Clark, M.; Witherspoon, D. *Newsweek* **1983**, *101*(18), 85.
Dodds, J. H.; Roberts, L. W. *Experiments in Plant Tissue Culture*; Cambridge University: Cambridge, 1982.
Food Chemicals Codex; National Academy of Sciences, National Research Council: Washington, DC, 1966; pp 488–490.
Fox, P. F.; Morrissey, P. A. In *Industrial and Clinical Enzymology*; Vitale, L. J., Simon, V., Eds.; Pergamon Press: Oxford, 1980; Vol. 61, p 43.
Graham, D. Y. In *Enzymes as Drugs*; Holcenberg, J. S., Roberts, J., Eds.; Wiley: New York 1981; pp 331–351.
Gunby, P. *JAMA, J. Am. Med. Assoc.* **1983**, *249*, 115.
Harvard Medical School Health Letter **1983**, *8*, 5.
Klausner, A. *Bio/Technology* **1983**, *1*, 734.
Knorr, D.; Teutonico, R. A. *J. Agric. Food Chem.* **1986**, *34*, 96.
Knorr, D.; Miazga, S. M.; Teutonico, R. A. *Food Technol.* **1985**, *39*(10), 135.
Liener, I. E. In *Food Related Enzymes*; Whitaker, J. R., Ed.; Advances in Chemistry Series 136; American Chemical Society: Washington, DC 1974; pp 202–217.
Löffler, A. *Food Technol.* **1986**, *40*(1), 63.
Lynn, K. R.; Yaguchi, M.; Roy, C. *Biochim. Biophys. Acta* **1980**, *624*, 579.
Mathews, V. H.; Rangan, T. S.; Narayanaswamy, S. *Z. Pflanzenphysiol.* **1976**, *79*, 450.
Medora, R. S., Campbell, J. M.; Mell, G. P. *Lloydia* **1973**, *36*, 214.
Murashige, T.; Skoog, F. *Physiol. Plant* **1962**, *15*, 473.
Peterson, M. S.; Johnson, A. H. *Encyclopedia of Food Science*; AVI: Westport CT, 1978; p 94–96.
Sahai, O. P.; Knuth, M. *Biotechnol. Prog.* **1985**, *1*, 1.
Schwimmer, S. In *Source Book of Food Enzymology*; AVI: Westport, CT, 1981a; pp 481–496.
Schwimmer, S. In *Source Book of Food Enzymology*; AVI: Westport, CT, 1981b; pp 89–104.
Singh, R. J. *Z. Pflanzenzüchtung.* **1984**, *92*, 95.
Straus, J.; Campbell, W. A. *Life Sci.* **1963**, *1*, 50.
Towill, L. E.; Mazur, P. *Can. J. Bot.* **1975**, *53*, 1097.
Ward, O. P. In *Comprehensive Biotechnology*; Pergamon: Oxford, 1985; Vol. 3, pp 789–819.
Winnick, T.; Davis, A. R.; Greenberg, D. M. *J. Gen. Physiol.* **1940**, *23*, 275.
Wolnak, B. In *Enzymes*; Banehy, J. P., Wolnak, B., Eds.; Marcel Dekker: New York, 1980; pp 3–10.
Vorlop, K. D.; Klein, j. *Biotechnol. Lett.* **1982**, *3*, 9.
Young, D. H.; Köhle, H.; Kauss, H. *Plant Physiol.* **1982**, *70*, 1449.

Received for review October 31, 1985. Revised manuscript received August 28, 1986. Accepted January 29, 1987.

Phospholipases C and D in Rice Grains

Joseph Chrastil* and Frederick W. Parrish

Evidence for the relatively high activities of phospholipases C and D was found in rice grains. Both enzymes were present in both the outer and the inner layers of the brown rice. Kinetic analysis has shown that both enzymes acted independently, and no consecutive reaction chains have been found. Both enzymic reactions were of the first order, and no structural diffusion hindrance was found. The enzymes hydrolyzed not only different lecithins or cephalins but also phosphorylcholine, phosphatidic acids, and (*p*-nitrophenyl)phosphorylcholine. The specific rate constants with these substrates were similar but were higher with phospholipase D than with phospholipase C. The enzymes were still active at 70 °C. The activation energy of the phospholipase C was close to 41 000 J/mol.

Phospholipases (enzymes hydrolyzing lecithins and/or cephalins) are not identical with ordinary lipases. They were first found in snake, wasp, and scorpion venoms (Delezenne and Ledebt, 1910, 1911, 1912; Long and Penny, 1957). Phosholipases A and B hydrolyze lecithins or cephalins in positions 2 and 3, with the formation of the corresponding fatty acid and lysolecithin (lysocephalin). Phospholipase C hydrolyzes the phosphoric acid–glycerin bond on carbon 1 with the formation of phosphorylcholine, and phospholipase D attacks the phosphorylcholine bond with the release of free choline (Lowenstein, 1969). Except phospholipase D (by this definition), all phospholipases produce lyso compounds that are hemolytic and toxic once they reach the blood or brain (Delezenne and Ledebt, 1910, 1911, 1912; Contardi and Latzer, 1928; Zeller, 1952).

Phospholipids play many important roles in metabolism. They interact with proteins and constitute an integral part of cell membranes, mitochondrial and microsomal cytochrome redox systems, or signal transfer cells. Many of the phospholipid functions are still unknown, especially in plants, and future work will evidently reveal much more about their importance.

Phospholipases have been demonstrated in plants, but except phospholipase D, they have not been studied as extensively as human phospholipases. Contardi and Ercoli (1933) have demonstrated phospholipases in rice bran.

Southern Regional Research Center, U.S. Department of Agriculture—Agricultural Research Service, New Orleans, Louisiana 70179.

This article not subject to U.S. Copyright. Published 1987 by the American Chemical Society

Table I. Correction Factors[a] for Enzyme Stability at pH 5.5

time, h	4 °C	25 °C	40 °C	50 °C
1	0.995	0.991	0.953	0.921
2	0.991	0.982	0.907	0.845
3	0.987	0.974	0.863	0.773
4	0.982	0.965	0.821	0.704
5	0.978	0.957	0.781	0.683
6	0.975	0.949	0.743	0.576
7	0.971	0.941	0.706	0.518
8	0.967	0.933	0.671	0.462
24	0.933	0.832	0.358	0.041
30	0.922	0.750	0.252	
48	0.882	0.580	0.098	
72	0.816	0.516		

[a] The values in the table are the correction factors z for the time curves $t_{cor} = zt_{expti}$. The z values were calculated as the integral middle values from the enzyme stability experiments.

Much later, phospholipase C in rice bran was studied by Takano et al. (1984). Kinetic properties of phospholipases in plants have not been studied in detail, and further investigation is deemed necessary.

EXPERIMENTAL SECTION

Source of Phospholipase Activity. On the basis of our preliminary experiments it became apparent that diluted NaOH was one of the best extraction mediums for different enzymes, included phospholipases, from rice grains. The extraction was accomplished in a reasonable time interval, and the final enzymic extract was close to pH 7. Brown medium-long rice (Mars) (or 30% polished rice) was milled to a flour, which was extracted with 0.01 M NaOH (1 + 2, w/v) by shaking for 30 min at 25 °C. The suspension was immediately centrifuged at 40000g (10 min) at 4 °C. The supernatant was filtered and used as an enzyme source for all experiments (final pH 7.0). Except the data in Table IV, all data in this work relate to the brown rice grains from medium rice variety (Mars).

Optimum pH. The optimum pH curves were determined from the initial rates of the phospholipases C and D reactions with different substrates at different pH in 0.2 M buffers (citrate, Tris, glycine) at 25 °C. Both phospholipases C and D had similar optima pH at 5.5.

Stability of Enzyme Extracts. In order to obtain correct time curves, we must know the stability of the enzyme extracts in the reaction mixture. The enzyme extracts were incubated in 0.2 M citrate buffer (pH 5.5) at different temperatures for selected time intervals. After the incubation the extracts were used immediately for the estimation of initial velocities of phospholipase reactions. The activity losses were expressed relative to the initial enzyme activity (initial enzyme activity 1), and the second-order regression time curves were calculated for every temperature to fit experimental results. The regression equations were integrated, and the integral middle values were calculated on the time limits $0 \rightarrow t$. In this manner we obtained the enzyme stability corrections (see Table I).

Protein. Protein was determined by the method of Lowry et al. (1951). Standardization was performed with gluten standards.

Phospholipase C. Phospholipase C activity was determined by p-nitrophenol (PNP) released from (p-nitrophenyl)phosphorylcholine (PNPPCH). PNPPCH stock solution (0.1 mL) was mixed with 2.4 mL of citrate buffer (0.2 M, pH 5.5) and 0.5 mL of enzyme extract. The final concentrations of PNPPCH in the incubation mixtures are shown in tables and figures. The reaction mixtures were incubated by shaking on a Nutator mixer. After the selected time interval 0.1 mL of the reaction mixture was added to 3 mL of 0.33 M NaOH, and the absorbance was read at 400 nm vs. H_2O. The standard was prepared from pure PNP (Sigma Chemical Co.). The action of phospholipase C on lecithin, cephalin, lysolecithin, or phosphatidic acid (PA) was indirectly determined by the simultaneous analysis of cholin (or ethanolamine) and free H_3PO_4.

Phospholipase D. The activity of phospholipase D was determined by free choline and/or H_3PO_4 in the incubated mixtures. The stock solution of PNPPCH or phosphorylcholine (PCH) in water (0.2 mL) or the solution or suspension of lecithin, lysolecithin, or cephalin in ether (1 mL) was mixed with 2.3 mL of acetate buffer (0.2 M, pH 5.5) and 0.5 mL of enzyme extract. The final concentrations of the substrates are shown in tables or figures. The reaction mixture was incubated by shaking on a Nutator mixer for selected time periods.

Free phosphoric acid was determined as follows: A 50-μL portion of the incubation mixture was mixed with 1 mL of acetate buffer (0.2 M, pH 5.5), 1 mL of 10% trichloroacetic acid, 1 mL of ammonium molybdate (5%), and 0.1 mL of ANSA (0.25 g of 1,2,4-triaminonaphtholsulfonic acid + 15 g of $NaHSO_3$ + 4 g of Na_2SO_3 in 100 mL of H_2O). The solution was heated for 15 min at 100 °C and then cooled for 45 min to room temperature (25 °C). The absorbance was read at 820 nm vs. H_2O. The standard was prepared from analytical NaH_2PO_4. The reaction blanks were determined at zero incubation times.

Choline was determined in the same incubation mixture as follows: A 2-mL sample of the incubation mixture was mixed with 1 mL of 10% TCA and the resultant mixture centrifuged. Of the supernatant, 2 mL was mixed with 1 mL of H_2O, 1 mL of 1 M NaOH, and 1 mL of Reinecke salt (4% in MeOH). The reaction mixture was left at 4 °C for 24 h and then centrifuged. The supernatant was discarded, and the pellet was washed with 3 mL of cold water and centrifuged again. Finally the pellet was dissolved in 3 mL of acetone and centrifuged, and the absorbance of the supernatant was read at 526 nm vs. H_2O. The standard was prepared from pure choline. All experiments were done in duplicate.

RESULTS AND DISCUSSION

Kinetic Analysis. The kinetic properties of phospholipases C and D were studied by means of complete time curves as described by Chrastil and Wilson (1982), by the statistical regression analysis of eq 1, where P is the

$$P = P_\infty[1 - \exp(-kEt)]^n \quad (1)$$

product of the reaction formed in time t, P_∞ is the equilibrium product concentration at time $t = \infty$, k is the specific rate constant, E is the enzyme concentration in the reaction mixture (expressed as protein concentration in grams per liter), and n is the diffusion resistance constant, which depends on the heterogeneous structure of the reaction system. With all enzymic systems and substrates studied in this work, n was close to 1 (between 0.98 and 1) and thus the reactions were of the first order.

The activation energies were calculated from the rate constants at different temperatures (25, 40, 50 °C) by means of the linear regression analysis of ln k vs. $1/T$ from eq 2 and 3, where T is the absolute temperature and R is the gas constant (in joules).

$$\ln k = a/T + b \quad (2)$$

$$\epsilon = -aR \quad (3)$$

The Henry–Michaelis–Menten constants were calculated by the statistical linear regression analysis of eq 4, where

Table II. Kinetic Constants for the Reaction of Phospholipase C from Rice Grains with PNPPCH[a]

S, M	temp, °C	$P_\infty \times 10^5$, M	$k \times 10^2$, $h^{-1}\ g^{-1}\ L$	r
1×10^{-4}	25	9.89	2.41	0.9996
4×10^{-4}	25	38.1	2.22	0.9961
1×10^{-3}	25	89.4	2.26	0.9984
1×10^{-3}	40	90.1	5.08	0.9993
1×10^{-3}	50	92.3	8.04	0.9949
4×10^{-3}	25	291	2.20	0.9997
1×10^{-2}	25	575	2.12	0.9971
4×10^{-2}	25	1420	2.03	0.9975

[a] Protein in reaction mixture, E = 1.35 g/L; S = PNPPCH; reaction product P = PNP; $V_{max} = 2.11 \times 10^{-4}$ M h^{-1}; $K_m = 6.60 \times 10^{-3}$ M; r = 0.9995; P_∞, equilibrium constant; k, specific rate constant; $k = \exp(-4909.1/T + 12.6827)$; r = 0.9998; ϵ = 41050 J mol^{-1}; r = statistical correlation coefficient.

Table III. Kinetic Constants for the Reaction of Phospholipase C from Rice Grains with PA[a]

S, M	$P_\infty \times 10^3$, M	$k \times 10^2$, $h^{-1}\ g^{-1}\ L$	r
1×10^{-3}	0.90	2.14	0.9990
5×10^{-3}	3.49	2.23	0.9919
1×10^{-2}	5.81	2.29	0.9985

[a] Protein in the reaction mixture, E = 1.35 g/L; S = PA; temperature, 25 °C; reaction product P = H_3PO_4; $V_{max} = 4.78 \times 10^{-4}$ M h^{-1}; $K_m = 1.76 \times 10^{-2}$ M; r = 1.0000; r = statistical correlation coefficient.

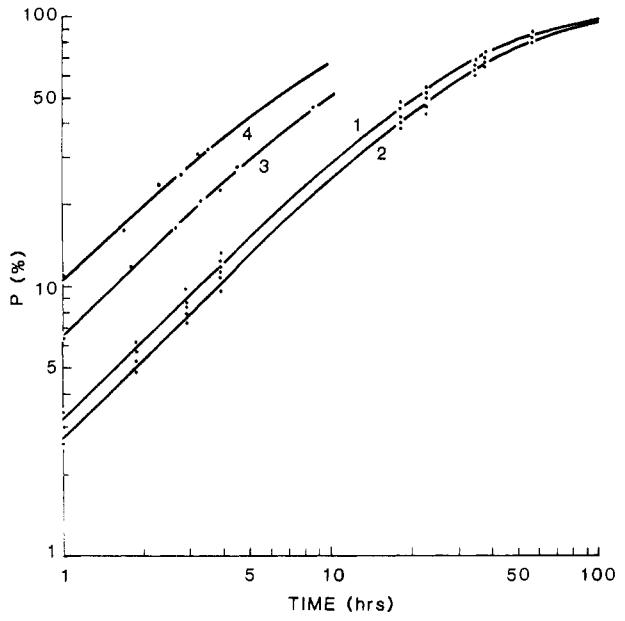

Figure 1. Hydrolysis of PNPPCH to PNP by phospholipase C from rice grains: (1) $S = 1 \times 10^{-4}$ M, 25 °C; (2) $S = 4 \times 10^{-2}$ M, 25 °C; (3) $S = 1 \times 10^{-3}$ M, 40 °C; (4) $S = 1 \times 10^{-3}$ M, 50 °C. The curves with other substrate concentrations were between 1 and 2. Points are experimental values, the curves are the calculated first-order reactions, respectively.

V_0 is the initial velocity of the reaction, S is the initial substrate concentration, and V_{max} and K_m are constants.

$$V_0 = V_{max}S/(K_m + S) \quad (4)$$

In all cases, the complete time curves were obtained and the first-order kinetic constants were calculated by the statistical regression. The statistical correlation coefficients of these calculations are shown in the tables. The equilibrium concentrations P_∞ were read from the time curves at $t \to \infty$. The initial velocities were calculated from the first-order reaction constants ($V_0 = P_\infty kE$). The reaction times were corrected by the correction factors from Table I.

Table IV. Kinetic Constants for the Reaction of Phopholipase C from Different Parts of the Medium Brown Rice (Mars) with PNPPCH[a]

	E, g/L	$P_\infty \times 10^3$, M	$k \times 10^2$, $h^{-1}\ g^{-1}\ L$	r
brown rice	1.35	1.71	2.23	0.9992
polished rice (30%)	0.65	1.74	2.30	0.9985
bran-polish (30%)	3.02	1.73	2.20	0.9993

[a] S = PNPPCH (2×10^{-3} M); E = protein in reaction mixture; reaction product P = PNP; temperature, 25 °C; r = statistical correlation coefficient; P_∞ and k, first-order reaction constants.

Table V. Kinetic Constants for the Reaction of Phospholipase D from Rice Grains with PNPPCH[a]

S, M	4×10^{-2}	S, M	4×10^{-2}
P_∞, M	0.0397	r	0.9987
k, $h^{-1}\ g^{-1}\ L$	0.0299		

[a] S = PNPPCH; reaction product P = choline; temperature, 25 °C; protein in reaction mixture, E = 1.35 g/L; r = statistical correlation coefficient.

Table VI. Kinetic Constants for the Reaction of Phospholipase D from Rice Grains with Lecithin[a]

S, M	4×10^{-2}	S, M	4×10^{-2}
P_∞, M	0.0395	r	0.9988
k, $h^{-1}\ g^{-1}\ L$	0.0298		

[a] S = lecithin; reaction product P = choline; temperature, 25 °C; protein in reaction mixture, E = 1.35 g/L; r = statistical correlation coefficient.

Table VII. Kinetic Constants for the Reaction of Phospholipase D from Rice Grains with PCH[a]

	S, M		
	3×10^{-3}	1.5×10^{-2}	3×10^{-2}
P_∞, M	3.00×10^{-3}	1.50×10^{-2}	2.98×10^{-2}
k, $h^{-1}\ g^{-1}\ L$	0.0304	0.0296	0.0281
r	0.9999	0.9990	0.9997

[a] S = PCH; reaction product P = choline; temperature, 25 °C; protein in reaction mixture, E = 1.35 g/L; $V_{max} = 1.07 \times 10^{-2}$ M h^{-1}; $K_m = 2.57 \times 10^{-1}$ M; r = statistical correlation coefficient.

Phospholipase C. The enzymatic extracts from the rice grains hydrolyzed PNPPCH and PA to free PNP and/or H_3PO_4, respectively. Both of these reactions were first order. The specific rate constants k were close to 0.022 ($h^{-1}\ g^{-1}$ L) for both PNPPCH and/or PA (Tables II and III; Figure 1). The activation energy of the PNPPCH reaction was ϵ = 41050 J mol^{-1} (Table II; Figure 2). The constant K_m for the reaction with PNPPCH was smaller than with PA, 6.60×10^{-3} and 1.76×10^{-2} M, respectively. This means that the enzyme had higher affinity to PNPPCH than to PA. It was found that some phospholipases were activated by sorbitol or other carbohydrates (Kurioka and Matsuda, 1976), but the reaction with the rice phospholipase C and PNPPCH was not activated by sorbitol (Figure 3).

The distribution of phospholipase C in brown rice depended on the rice variety, storage, and other factors. The enzymes in medium brown rice (Mars) were almost uniformly distributed (Table IV), but in most other varieties or after longer storage the enzymes were preferably concentrated in inner endosperm layers (not shown here). Phospholipase C in rice extract hydrolyzed not only PNPPCH and PA but also different kinds of lecithins,

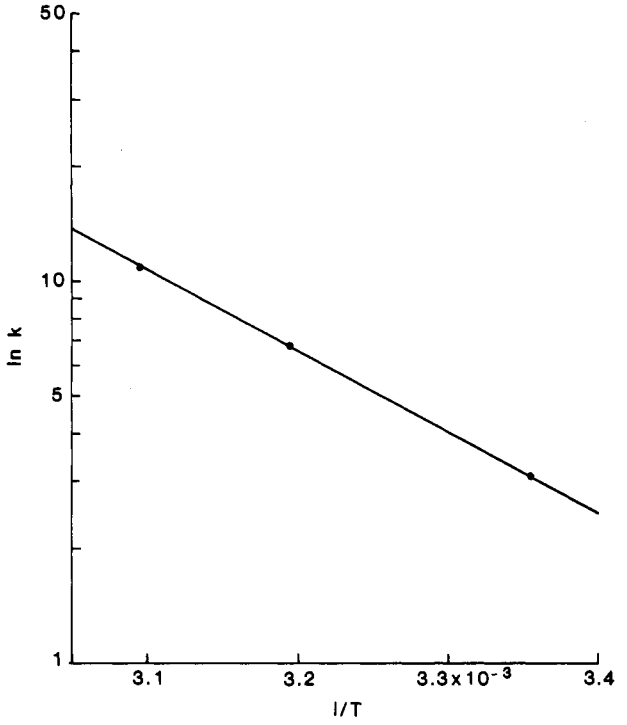

Figure 2. Activation energy of the hydrolysis of PNPPCH by phospholipase C from rice grains: $S = 1 \times 10^{-3}$ M; temperatures, 25, 40, and 50 °C.

lysolecithins, and cephalins.

Phospholipase D. The enzymatic extracts from the rice grains hydrolyzed PNPPCH or PCH and lecithins or cephalins almost quantitatively to free choline or ethanolamine. These phospholipase D reactions were all first order ($n = 1$). The hydrolysis of these substrates to free choline or H_3PO_4 by the rice extracts was faster than the hydrolysis of PNPPCH, PA, or lecithins to PNP and/or H_3PO_4 by the action of phospholipase C ($k = 0.030$ and 0.022 h^{-1} g^{-1} L, respectively) (Tables V–VII).

The constant K_m was determined only with PCH and was 2.57×10^{-1} M, and thus, although the reaction was faster than the hydrolysis by phospholipase C, the affinity of phospholipase D to PCH was lower than the affinity, for example, of phospholipase C to PNPPCH, and/or PA.

Phospholipase D was found in all parts of the brown rice, and it was distributed in manner similar to that of phospholipase C (not shown here). The specific rate constants k obtained by the reaction of the rice extracts with other substrates (natural or synthetic lecithins and cephalins) were all close to 0.030 h^{-1} g^{-1} L.

Both phospholipase C and D specifically hydrolyzed the corresponding bonds of the phospholipids and were not influenced by other groups of the phospholipid molecules. Both acted independently and simultaneously, and no consecutive reaction chain could be found.

The rice phospholipases hydrolyzed not only different lecithins or cephalins but also phosphorylcholine, phosphatidic acids, and (*p*-nitrophenyl)phosphorylcholine. Lecithins and cephalins were hydrolyzed with similar rates. The specific rate constants of the phospholipase D reaction with the same rice extract were always higher than the specific rate constants of the phospholipase C reaction.

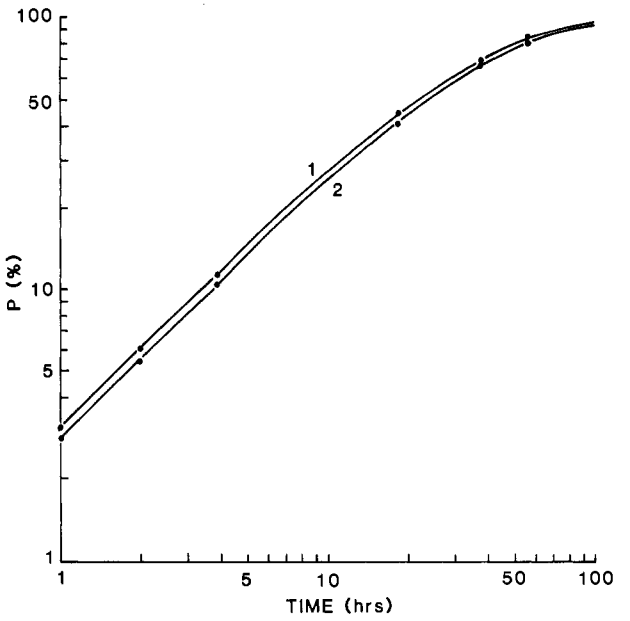

Figure 3. Hydrolysis of PNPPCH to PNP by phospholipase C from rice grains in the presence of 60% (w/v) sorbitol ($S = 2 \times 10^{-3}$ M; $P_\infty = 1.65 \times 10^{-3}$ M, 25 °C): (1) control without sorbitol, $k = 0.0232$ h^{-1} g^{-1} L; (2) control with 60% sorbitol (w/v), $k = 0.0215$ h^{-1} g^{-1} L. Points are experimental values, and curves are the calculated first-order reactions, respectively.

The enzymes were still active at 70 °C. The activation energy of phospholipase C was close to 41000 J mol^{-1}.

In the same rice grains used for the experiments with phospholipase C and D, we have also confirmed the presence of phospholipases A and B (not shown here). Both of these reactions were also of the first order with the specific reaction rates comparable to those of the phospholipases C and D. There was no significant difference between the kinetic properties of phospholipases in the extracts from brown rice, polished (30%) rice, or bran-polish (not shown here).

LITERATURE CITED

Chrastil, J.; Wilson, J. T. *Int. J. Biochem.* **1982**, *14*, 1.
Contardi, A.; Latzer, P. *Biochem. Z.* **1928**, *197*, 222.
Contardi, A.; Ercoli, A. *Biochem. Z.* **1933**, *261*, 275.
Davidson, F. M.; Long, C. *Biochem. J.* **1958**, *69*, 458.
Delezenne, C.; Ledebt, S. *C. R. Hebd. Seances Acad. Sci.* **1910**, *152*, 790; **1911**, *153*, 81; **1912**, *155*, 1101.
Hanahan, D. J.; Chaikoff, I. L. *J. Biol. Chem.* **1947**, *169*, 699; **1948**, *172*, 191.
Hanahan, D. J.; Rodbell, M.; Turner, L. D. *J. Biol. Chem.* **1954**, *206*, 431.
Kates, M. *J. Biochem. Physiol.* **1955**, *33*, 575.
Kurioka, S.; Matsuda, M. *Anal. Biochem.* **1976**, *75*, 281.
Long, C.; Penny, I. F. *Biochem. J.* **1957**, *65*, 382.
Lowenstein, J. M. *Methods Enzymol.* **1969**, *XIV*.
Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
McFarlane, M. G.; Knight, B. C. J. G. *Biochem. J.* **1941**, *35*, 884.
Takano, K.; Kamoi, I.; Obara, T. *Nokagu Shuho* **1984**, *28*, 262.
Zeller, E. A. *Fed. Proc.* **1952**, *11*, 316.

Received for review June 16, 1986. Revised manuscript received November 6, 1986. Accepted April 20, 1987.