KOREAN J. FOOD SCI. TECHNOL.
Vol. 16, *No.* 2 (1984)

Purification and Characterization of Chinese Cabbage Pectinesterase

Young-Hwan Ko and Kwan-Hwa Park

Department of Food Science and Technology, Seoul National University, Suwon

배추 펙틴에스테라제의 정제 및 특성

고영환 · 박관화

서울대학교 농과대학 식품공학과

Abstract

Two fractions of pectinesterase from Chinese cabbage were isolated by ammonium sulfate fractionation, ion exchange chromatography on DEAE-cellulose and Sephadex G-150 gel filtration. The fraction F-A and F-B were purified approximately 340- and 10-fold. The similar salt effects and pH optima (pH 7.5-8.0) were obtained for the two pectinesterase fractions. The maximum activity of both two fractions were obtained at 20-50*mM* of divalent cations and at 250*mM* of monovalent cations. The apparent Michaelis constant of the F-A was 0.01% for citrus pectin. The temperature optima for F-A and F-B were 48° and 55°C, respectively and both fractions were stable in the region of pH 5.0-8.0 at room temperature. The thermal inactivation of the two fractions followed the first order reaction kinetics. From D and Z-values obtained the thermal resistance of the two fractions were characterized.

Introduction

Pectinesterase (Pectin pectyl hydrolase, EC, 3.1.1.11) catalyzes the deesterification of the methyl ester groups of pectin which are adjacent to free carboxyl group. The action of the enzyme results in a decreased solubility of pectic substances, particularly in the presence of calcium salts, and also an increased firmness of tissues. When plant tissue is held at moderate temperature (40-70°C) and subsequently boiled, it attains a firmer texture than the corresponding untreated control.[1] Many works on the pectinesterase have been reported in connection with texture of potatoes,[2] cauliflowers,[3] carrots,[4] cucumbers,[5,6] tomatoes[7,8] and Japanese radish roots.[1]

Chinese cabbage is one of the popular vegetables in oriental area and mainly used as a fermented product (e.g. *Kimchi*) or salads. Very few studies have been reported on the texture of Chinese cabbage during fermentation.[9] Moreover little information is available on pectinesterase from Chinese cabbage. This paper presents the results of purification of pectinesterases from Chinese cabbage and the properties of the pectinesterase in order to elucidate the kinetic behaviour of pectinesterase isozymes.

Materials and Methods

Pectinesterase Assay

Chinese cabbage was purchased from a local market in Suwon, Korea. The pectinesterase activity was measured titrimetrically by a modified method of Kertesz.[10]

Fifteen hundredth mole of NaOH solution was added to 50*ml* of 0.45% pectin solution(Chin Hwa Pharm. Co., Korea) containing 0.15*mM* NaCl to adjust pH to 7.0 and

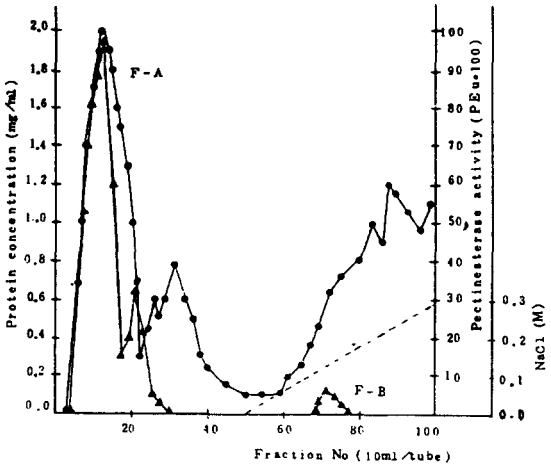

Fig. 1. Chromatography of Chinese cabbage pectinesterase on DEAE-Cellulose
●—● Protein; ▲—▲ Pectinesterase activity; ··· NaCl

0.2*ml* of enzyme solution was added. Preincubation was followed at 30°C for 5 min. The reaction mixture was stirred with magnetic strirrer, titrated continuously to maintain pH at 7.0 with 0.02*N* sodium hydroxide during incubation. One unit of pectin-esterase activity (PEu) was defined as the amount of enzyme which produces 2×10^{-5} moles of free carboxyl group per min under the assay condition.

Protein was determined by the method of Lowry et al.[11] using bovine serum albumin as a standard protein.

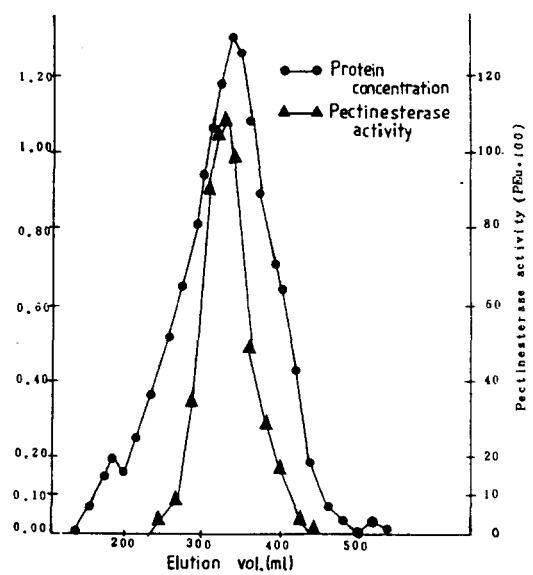

Fig. 2. Chromatography of F-A on Sephadex G-150

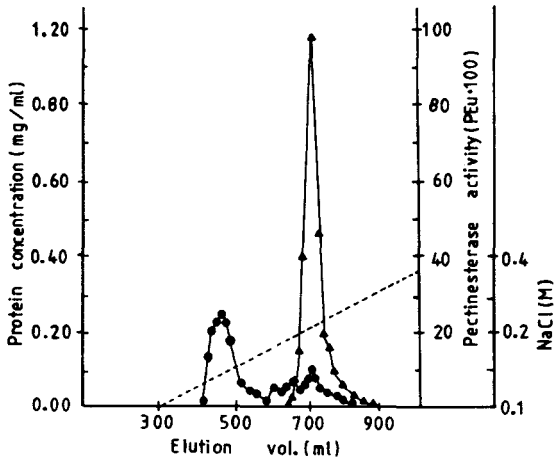

Fig. 3. Chromatography of F-A on CM-Cellulose
●—● Protein; ▲—▲ Pectinesterase activity; ··· NaCl

Purification

Chinese cabbage was sliced to small pieces. One liter of 0.2*M*, pH 8.0 phosphate buffer per *kg* of plant material was added and the mixture was homogenized with Waring blendor for 20-30 sec. After standing for 24 hr the suspension was pressed through the double layers of nylon cloth and then centrifuged for 15 min at 9,000 × g. The supernatant was used as the crude extract of the enzyme. Solid ammonium sulfate was slowly added to the extract to 40% saturation with gentle stirring. The mixture was standed for 3 hr and then centrifuged for 15 min at 12,000 × g. Solid ammonium sulfate was further added to the supernatant to 70% saturation, and then the

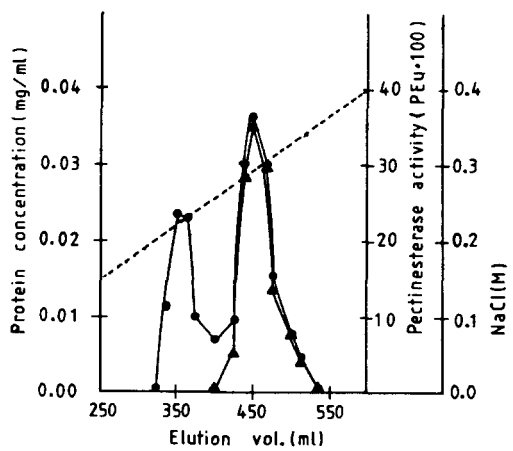

Fig. 4. Rechromatography of F-A on CM-Cellulose
●—● Protein; ▲—▲ Pectinesterase activity; ··· NaCl

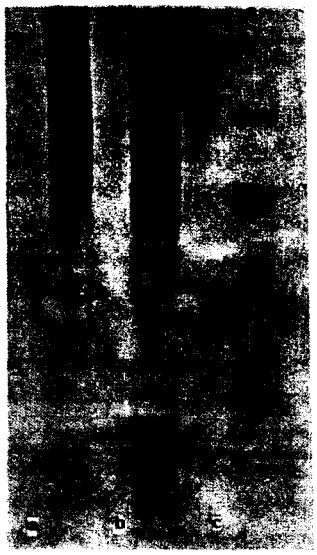

Fig. 5. Electrophoretic pattern of pectinesterase on polyacrylamide gel(15%) at pH 4.3
a. 40~70% ammomium sulfate precipitate of crude extract
b. F-A from DEAE-cellulose
c. F-A from CM-cellulose rechromatography

mixture was recentrifuged. The precipitate was dissolved in a small amount of distilled water and dialyzed against 0.01*M*, pH 7.5 phosphate buffer for 24hr.

The dialyzate was applied to a DEAE-cellulose column (2.5×25*cm*) which had been equilibrated with 10*mM* phosphate buffer, pH 7.5. The column was eluted with the same buffer at a flow rate of 30*ml* per hour until the activities of the first part of enzyme (fraction A) were

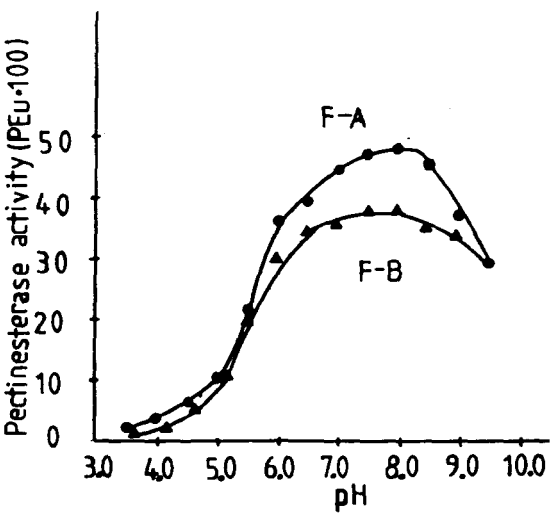

Fig. 6. Effect of temperature on pectinesterase activity

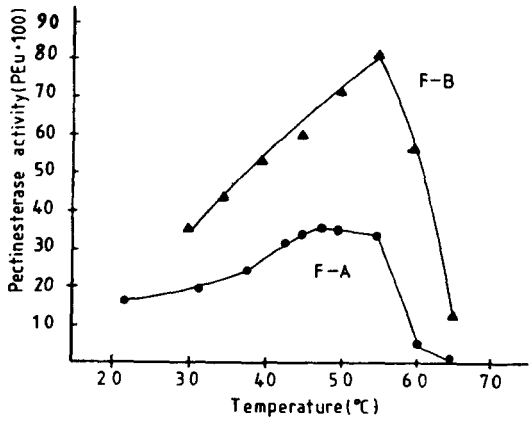

Fig. 7. Effect of temperature on pectinesterase activity

detected and then with the NaCl solution in 10*mM* phosphate buffer, pH 7.5. Fraction of 10*ml* each were collected and the fractions containing pectinesterase activity were pooled and brought to 70% saturation by addition of ammonium sulfate. Precipitate was obtained with centrifugation and redialyzed.

The redialyzate of fraction A was applied to a Sephadex G-160 column (2.5×75*cm*) preequilibrated with 10*mM* phosphate buffer, pH 7.5 and the column was eluted with the same buffer. The flow rate was 20*ml* per hour. Pectinesterase active fractions were combined again, concentrated with gum arabic, and dialyzed in 10*mM* phosphate buffer, pH 7.5.

The dialyzate was concentated with gum arabic powder and rechromatographed on CM-cellulose column

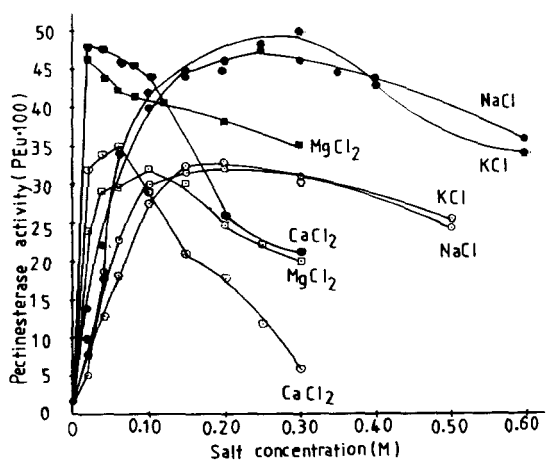

Fig. 8. Effect of salts on pectinesterase activity
Closed ; F-A fraction;
Open ; F-B fraction

chromatography. The tubes containing active fractions were pooled finally and preserved in the refrigerator for further use.

Polyacrylamide Gel Electrophoresis

Samples containing 30-200 μg of protein were subjected to disc electrophoresis. Electrophoresis on 7% and 15% polyacrylamide gel was carried out at pH 8.9 by the method of Davis[12] and at pH 4.3 by the method of Reisfeld et al.[13]

Thermal Inactivation

Nine mililiters of 10mM phosphate buffer, pH 7.5 was added to a 12×100mm test tube, and then the buffer solution ws equilibrated to heating temperature in a water bath. One mililiter of enzyme solution was added to the buffer solution and the mixture was stirred continuously with magnetic stirrer during the heating process. Aliquots of 0.5ml were taken at various time intervals and immediately cooled in an ice water bath. Then the residual enzyme activity was measured.

Results and Discussion

Purification of Pectinesterase

Two enzyme activity peaks, fraction A and fraction B were shown in the elution profile (Fig. 1). Fraction A (F-A), the major portion of pectinesterase activity, was eluted from the DEAE-cellulose ion exchanger. Hultin et al.[14] reported that at least three fractions of pectinesterase were identified from the banana pulp by differential extraction procedures. Nakagawa et al.[15] reported that two fractions of pectinesterase were isolated from tomato by DEAE-cellulose ion exchange column chromatography. The tomato pectinesterase elution profile is similar to our result. Versteeg et al[16] reported that two isozymes were purified from orange by gel chromatography. Körner et al.[17] also isolated two orange pectinesterases by ammonium sulfate fractionation and ion exchange chromatography.

Fraction A(F-A) was applied to the further purification. The peak of pectinesterase active fraction corresponded to that of protein on Sephadex G-150 column

Table 1. Purificatien of Chinese cabbage pectinesterase

Step of Purification	Volume (ml)	Total activity (PEu)	Total protein (mg)	Specific activity (PEu/mg)	Yield (%)	Fold
Crude extract	3,500	490.0	14,000	0.035	100	1
First 40-70% $(NH_4)_2SO_4$ saturation	60	201.6	600.0	0.336	41.1	9.6
DEAE-cellulose chromatography						
F-A	340	125.5	88.4	1.416	25.5	40.5
F-B	130	15.5	41.6	0.375	3.2	10.7
Second 40-70% $(NH_4)_2SO_4$ saturation						
(F-A)	20	66.0	72.0	0.916	13.5	26.2
CM-cellulose chromatography						
(F-A)	140	28.0	2.8	10.00	5.7	285.7
Concentration with gum arabic						
(F-A)	40	20.8	2.6	8.00	4.2	228.6
CM-cellulose rechromatography						
(F-A)	106	19.08	1.59	12.00	3.9	342.8

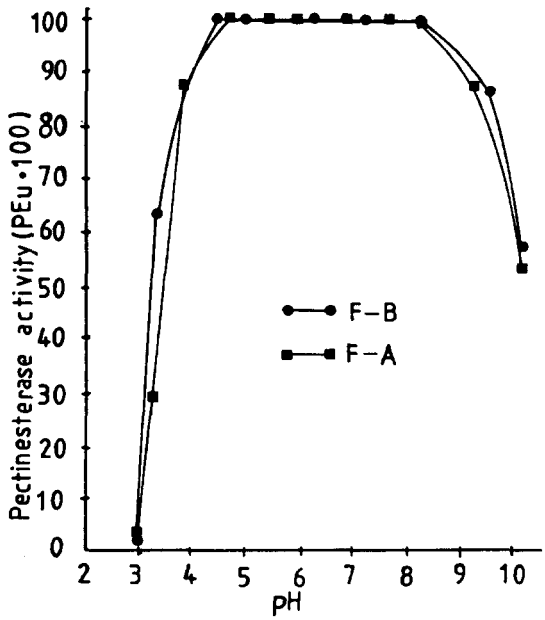

Fig. 9. pH stability of pectinesterase

chromatogram (Fig. 2). As shown in Fig. 3, pectinesterase was eluted at 0.25*M* NaCl concentration.

On the second CM-cellulose column chromatography was obtained as shown in Fig. 4. The pectinesterase active fraction coincided with protein peak.

A summary of the entire purification is presented in Table 1. First ammonium sulfate precipitation increased the purity of the enzyme 9.6 fold. Chromatography on DEAE-cellulose increased the purity 10.7 fold for F-B. The F-A was purified approximately 340 fold through the purification procedure.

Polyacrylamide Gel Electrophoresis

To examine the purity of enzyme solutions, electrophoresis was run at two pH systems and two gel concentrations. The electrophoretograms are shown in Fig. 5. Precipitate of crude extract of 40-70% ammonium sulfate saturation was resolved into several protein bands on polyacrylamide gel (Fig. 5a). One protein band was observed in the final preparation of F-A (Fig. 5c).

Properties of Pectinesterase Fractions

pH optimum for the activity: Non-enzymatic deesterification was determined at each pH value in the absence of pectinesterase, and corrections were made. pH optima were observed around 8.0 for the F-A (Fig. 6). The pH optimum was broadened from 6.5 to 8.5 for the

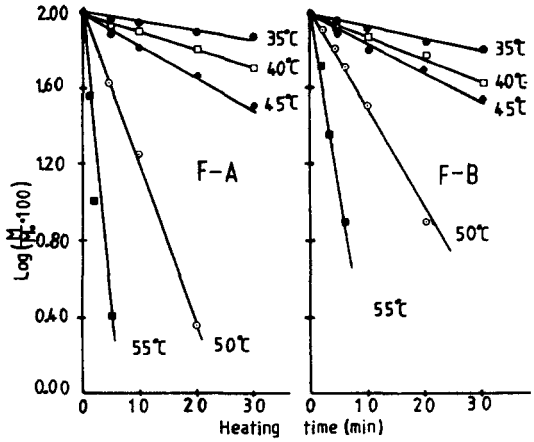

Fig. 10. Thermal inactivation of pectinesterase as a funtion of heating time

fraction F-B. Most of pectinesterases of higher plants have their pH optima in the range of pH 7.0-9.0.[14,17]

Effect of temperature: The initial reaction rate was determined at various temperatures. Non-enzymatic deesterification was also determined at each temperature in the absence of pectinesterase, and corrections were made.

As shown in Fig. 7, maximum reaction rate was obtained at 48 ℃ and 55 ℃ for F-A and F-B, respectively. According to Körner et al.,[17] two orange pectinesterases showed temperature optima at 60 ℃.

Effect of salts: Divalent cations such as Ca^{++} and Mg^{++} were effective on pectinesterase activity than monovalent cations such as K^{+} and Na^{+} (Fig. 8). Both fractions F-A and F-B had a maximum reaction rate in the range of 20-50*mM* of divalent cations, while higher concentrations of 200*mM* was necessary for maximum activity, and the inhibition did not occur drastically at higher concentration.

The effect of salts on the enzyme activity is pH-dependent. MacDonnell et al.[18] found the greater influence of divalent cations as compared with monovalent cations on the activity-pH relationship of orange pectinesterase. The reaction rate was measured only at pH 7.0 in our experiment. However, the effect of salts was found to be similiar to that reported.[18,19]

Effect of substrate concentration: The effect of citrus pectin concentration on pectinesterase activity was determined by measuring the initial reaction rates at various concentrations of pectin. An apparent Michaelis constant (*Km*) of F-A obtained for citrus pectin was

Table 2. First order reaction rate constants, D- and Z-values for inactivation of pectinesterase fractions

Heating temp. (℃)	D-value (sec)		Reaction rate constant, k ($sec^{-1} \times 10^4$)		Z-value (℃)	
	F-A	F-B	F-A	F-B	F-A	F-B
35	9230	60000	2495	0.384		
40	4067	5714	5.661	4.030		
45	2891	4210	7.964	5.470	10.5	8.3
50	794	1100	28.979	20.909		
55	141	24	163.129	107.473		

Table 3. Thermodynamic constants for inactivation of pectinesterase fractions

Temperature (℃)	$\Delta H^{\neq}$ (*kJ/mole*)		$\Delta G^{\neq}$ (*kJ/mole*)		$\Delta S^{\neq}$ (*J/mol. K*)	
	F-A	F-B	F-A	F-B	F-A	F-B
50	163	233.4	95.04	95.92	210.4	425.6

0.01% which is lower compared with the *Km* value of 0.24% for tomato pectinesterase[19] and that of 0.029-0.031% for orange pectinesterases.[17]

pH stability: Enzyme solution was incubated for 1 hr at various pH values and the residual activity was analyzed at pH 7.0. Fig. 9 illustrates the both pectinesterase fractions were stable in the region of pH 7.5-8.0.

Thermal stability: The heat stability was determined in the temperature range of 35-55 °C. The thermal inactivation of the fractions followed the first order reaction kinetics (Fig. 10). Decimal reduction time, D and reaction rate constant, *k* were calculated from these inactivation curves and summarized in Table 2. Thermodynamic data obtained at 50 °C are also summarized in Table 3. As shown in Table 2, D-value of the F-B was larger than that of the F-A. F-B was more heat-resistant than F-A in the range of temperature 35 °-55 °C. Z-value of the former was smaller than that of the latter.

Versteeg et al.[16] reported that three isozymes from orange showed different thermostability; Pectinesterase II was rapidly inactivated at 60 °C, Pectinesterase I at 70 °C, and Pectinesterase III at 90 °C, while Z-value were identical for two of the isozymes.[8]

In view of the importance of vegetable softening the heat stability of pectinesterase isozymes may be considered to constitute a potential problem to vegetable process industry.

요 약

배추로부터 얻은 펙틴에스테라제 조효소액을 DEAE-셀룰로오스 이온교환 수지를 통과시켜 두개의 분획F-A와 F-B를 분리했으며, 그중 주종을 이루고 있는 F-A는 DEAE-셀룰로오스 이온교환수지, CM-셀룰로오스 이온교환수지를 통과시켜서 약 340배 정제하였다. F-A와 F-B는 비슷한 염효과를 나타내었는데, 최적 염농도는 Ca^{++}, Mg^{++}등 2가 양이온의 경우 20~50*mM* K^+, Na^+등 1가 양이온의 경우 250*mM* 부근이었다. 최적pH는 F-A와 F-B에 대해서 각각 8.0 및 7.5~8.0이었으며, 이들 두 분획은 pH 5.0~8.0에서 적어도 한 시간동안 안정했다. F-A에 대한 *Km*은 펙틴기질에 대해서 0.01%이었다. F-A와 F-B의 최적온도는 각각48 ℃와 55℃이었으나 55℃에서 급격히 역가가 감소되었다. 열불활성화 경향은 1차반응을 나타내었으며 35℃~55℃사이에서 F-B의 D-Value는 F-A보다 커서 F-B가 F-A보다 열에 더 안정한 것으로 나타났다. 한편 Z-value는 F-A의 경우 10.5℃, F-B의 경우 8.3 ℃이었다.

References

1. Manabe, T.: *Nippon Shokuhin Kogyo Gakkaishi*, 27, 234 (1980)
2. Bartolome, L. G. and Hoff, J. F.: *J. Agr. Food Chem.*, 20, 266 (1972)
3. Hoogzand, C. and Doesburg, J. J.: *Food Tech.*, 15, 160 (1961)
4. Lee, C. Y., Bourne, M. C. and Van Buren, J. P.: *J. Food Sci.*, 44, 615 (1979)
5. Bell, T. A. and Etchells, J. L.: *J. Food Sci.*, 26, 84 (1961)
6. Demain, A. L. and Phaff, H. J.: *J. Agri. Food Chem.*, 5, 60 (1957)
7. Deshapande, S. N., Klinker, W. J., Draudt, H. N. and Desrosier, N. W.: *J. Food Sci.*, 27, 594 (1962)
8. Versteeg, C., Rombouts, F. M., Spansen, C. H. and Pilnik, W.: *J. Food Sci.*, 45, 969 (1980)
9. Ha, S. S.: Bulletin of the Scientific Res. Inst., M. N. D. Korea, 5, 139 (1960)
10. Kertesz, Z. I.: In *"Methods in Enzymology"* (Colowick, S. P. and Kaplan, N. O., ed.) Vol. 1. p. 158, Academic Press, N. Y. (1955)

11. Lowry, O., Rosenbrough, O. H., Farr, N. Z. and Randall R. J. : *J. Biol. Chem.*, **193**, 265 (1951)
12. Davis, B. J. : *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964)
13. Reisfeld, R. A., Lewis, U. J. and Williams, D. E. : *Nature*, **195**, 281 (1962)
14. Hultin, H. O. and Levine, A. S. : *Arch. Biochem. Biophys.*, **101**, 396 (1963)
15. Nakagawa, H., Tanagawa, Y. and Takehana, H. : *Agri. Biol. Chem.*, **34**, 991 (1970)
16. Versteeg, C., Rombouts, F. M. and Pilnik, W. : *Lebensm. Wiss. Technol.*, **11**, 267 (1978)
17. Körner, B., Zimmermann, G. and Berk, Z. : *J. Food Sci.*, **45**, 1203 (1980)
18. Macdonnell, L. R., Jansen, E. F. and Lineweaver, H. : *Arch. Biochem.*, **6**, 389 (1945)
19. Nakagawa, H., Yanagawa, Y. and Takehana, H. : *Agr. Biol. Chem.*, **34**, 998 (1970)

(Received March 30, 1984)