Specificity for Incorporation of α-Amino Acid Esters during the Plastein Reaction by Papain

Keiichi Aso, Michiko Yamashita, Soichi Arai,* Junko Suzuki, and Masao Fujimaki

Ovalbumin was partially hydrolyzed with papain (EC 3.4.4.10) to obtain a peptide fraction with a molecular weight of 500–4500. A 30% (w/v) solution of this peptide in an aqueous medium, pH 6.0, was mixed with each of various α-amino acid esters so as to be 20 mM. Each mixture was subjected to the plastein reaction by papain in the presence of 10 mM L-cysteine at 37 °C. During the reaction, L-amino acid ethyl esters were incorporated with different initial velocities depending on their amino acid side chain structure; the velocities tended to increase with increasing hydrophobicities of the side chains, except for the cases of β branched-chain amino acids. Even poor hydrophobic and β branced-chain amino acids could be effectively incorporated when esterified with longer chain alcohols. Esterification of some L-amino acids with branched-chain alcohols did not affect their initial velocities of incorporation. No significant amounts of amino acid polymers were formed under the reaction conditions employed in the present study.

The plastein reaction has been studied in detail as seen from recent reviews (Arai et al., 1975; Eriksen and Fagerson, 1976; Yamashita et al., 1976a). This reaction may gain useful value when applied as a tool for incorporating some essential amino acids and thus making up plasteins whose amino acid compositions have been improved from those of the original proteins. Yamashita et al. (1971) first tried to incorporate L-methionine by means of the plastein reaction in order to enhance a sulfur-containing amino acid level of soy protein. Subsequently, similar trials have been made aimed at improving nutritional and functional properties of conventional food proteins (Aso et al., 1974; Yamashita et al., 1975) and at maximizing nutritive quality of unconventional proteins from photosynthetic origin (Arai et al., 1976). Such an enzymatic process can be applied also to preparing a dietetic food material with a controlled amino acid pattern (Yamashita et al., 1976b). All these studies have employed papain enzyme evaluated as the best for the purpose of incorporating amino acids as far as they are used in ethyl ester form. Enzymologically, papain (EC 3.4.4.10) has been well defined for its catalytic function to hydrolyze peptides, amides, and esters (Glazer and Smith, 1971). Also, this enzyme is known to catalyze unusual reactions such as transpeptidation (Glazer and Smith, 1971), transamidation (Mycek and Fruton, 1957; Durell and Fruton, 1954) and transesterification (Glazer, 1966; Lake and Lowe, 1966; Sluyterman and Wijdenes, 1972) which all can give rise to new bonds. Up to the present time, however, no comprehensive information has been obtained on a papain-catalyzed reaction between peptides and amino acid esters to form new peptide bonds, especially on such a reaction proceeding under the conditions for the plastein reaction in which an exceptionally high substrate concentration is required.

The present study attempts to find out how, during the plastein reaction, different α-amino acid esters added in this reaction system can react differently depending upon their amino acid side chain and alcohol chain structures, in order to get general information that will be applicable for controlled alteration of amino acid compositions of food proteins.

EXPERIMENTAL SECTION

Enzyme. A twice-crystallized preparation of papain (Sigma Chemical Co.; No. P-3125) was used both for preparing an ovalbumin hydrolysate as substrate and for producing a plastein from this substrate.

Substrate. A reagent grade preparation of ovalbumin (Difco) was dissolved in a large amount of 0.01 N NaOH and the solution allowed to stand for 12 h at room temperature. This was dialyzed in a cellophane tube against running water at 5 °C for 24 h. The nondiffusible fraction, after adjusted to pH 6.0 with HCl, was freeze-dried and then hydrolyzed with papain. This hydrolysis was carried out under the following conditions: ovalbumin concentration in reaction medium, 1% (w/v); papain–ovalbumin ratio, 1:1000 (w/w); pH of the reaction system, 6.0; temperature, 37 °C; and reaction time, 24 h. The reaction system contained 10 mM L-cysteine as activator. No appreciable change in pH was observed during the incubation. An entire reaction mixture after the incubation was heated at 95 °C for 10 min, clarified with a filter paper (Toyo No. 2) and in turn treated with an ultrafiltration membrane (Amicon UM-05) to remove salts, free amino acids, and some small peptides having molecular weights below 500. Disc electrophoresis on polyacrylamide gel (Work, 1964) demonstrated that the ovalbumin hydrolysate obtained through these procedures had a molecular weight of 500–4500. This fraction was freeze-dried and used as the substrate for the plastein reaction.

Amino Acid Esters. Crystallized α-amino acids (Tokyo Kasei Kogyo Co.) were esterified with ethanol by the method of Boissonas et al. (1956). The esterification with other alcohols was carried out according to the method of Cipera and Nicholls (1955). The amino acid esters prepared were purified by column chromatography on silica gel and crystallized from chloroform–hexane. The crystallization was repeated until each of the amino acid esters showed a single spot when checked by thin-layer chromatography on silica gel with 1-butanol–acetic acid–water (4:1:2) (Brenner et al., 1969). The observed R_f values (× 100) were as follows: ethyl esters of glycine, 51; L-alanine, 65; D-alanine, 65; α-methylalanine, 73; L-α-aminobutyric acid, 77; L-methionine, 87; L-valine, 90; L-norvaline, 90; L-isoleucine, 92; L-leucine, 92; L-norleucine, 92; L-tyrosine, 89; L-phenylalanine, 93; L-tryptophan, 95; *n*-butyl ester of L-alanine, 79; *n*-hexyl esters of glycine, 69; L-alanine, 85; α-methylalanine, 86; L-α-aminobutyric acid, 90; L-valine, 93; L-norvaline, 92; L-isoleucine, 94; L-leucine, 94; L-norleucine, 94; and *n*-octyl ester of L-alanine, 88. The R_f values (× 100) of glycine esterified with 1-pentanol, isopentanol, 2-methyl-1-butanol, *sec*-pentyl alcohol, and *tert*-butyl alcohol were 65, 64, 65, and 60, respectively, and those of L-norleucine esterified with the same alcohols were 92, 91, 92, and 92 in the same order.

Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

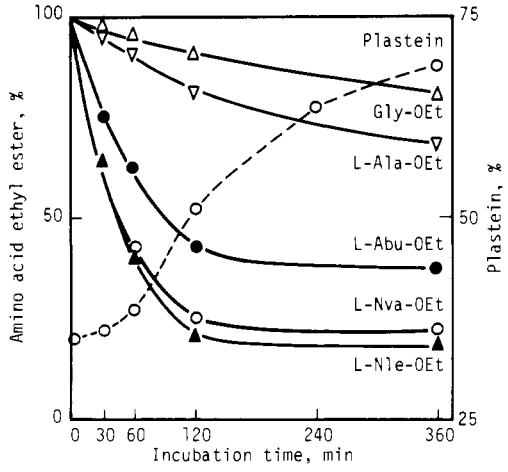

Figure 1. Decreases of glycine ethyl ester (Gly-OEt), L-alanine ethyl ester (L-Ala-OEt), L-α-aminobutyric acid ethyl ester (L-Abu-OEt), L-norvaline ethyl ester (L-Nva-OEt), and L-norleucine ethyl ester (L-Nle-OEt) during the plastein formation by papain from an ovalbumin hydrolysate. The plastein amount is given as: (CCl_3COOH-insoluble nitrogen/total nitrogen) × 100.

Plastein Reaction. The ovalbumin hydrolysate (300 mg) was dissolved in a small amount of water and adjusted to pH 6.0 with a very small amount of 2 N NaOH. The final volume was then adjusted to 0.9 mL with additional water. To the solution was added one of the amino acid esters so as to be 20 mM and the mixture preincubated at 37 °C for 15 min. On the other hand, papain (3 mg) was dissolved in 0.1 M L-cysteine (0.1 mL). This enzyme solution and the above-mentioned preincubation mixture were combined with each other, and the combined solution was incubated at 37 °C for a stated period. An aliquot of the incubation mixture was treated with CCl_3COOH according to the method of Yamashita et al. (1970) in order to quantify the plastein formed. A control experiment was carried out in which 20 mM amino acid ester alone was dissolved in 1 mL of L-cysteine (pH 6.0) containing 3 mg of papain and incubated at 37 °C.

Determination of Reactivities of Amino Acid Esters. Another aliquot (0.1 mL) of the incubation mixture was combined with 0.1 N NaOH (0.9 mL) and allowed to stand for 3 h at room temperature to complete the hydrolysis of the amino acid ester remaining unreacted. The free amino acid thereby produced was determined with an amino acid analyzer (Hitachi KLA-5). A reactivity of each amino acid ester was obtained by subtracting the determined value from the initial concentration (20 mM).

Determination of Amino Acid Composition. A plastein sample (1 mg) was hydrolyzed with 6 N HCl (1 mL) in an evacuated tube at 110 °C for 24 h. The hydrolysate was analyzed for amino acids with the amino acid analyzer. Half-cysteine was determined by the performic acid-oxidation method (Schram et al., 1954) and tryptophan by the method employing dimethylaminobenzaldehyde (Spies and Chambers, 1948).

Determination of Hydrophobicities. The hydrophobicities of amino acids relative to that of glycine were determined by means of paper partition chromatography using 70% (v/v) 1-propanol as solvent. The determination was conducted according to the method of Arai et al. (1973) which was based on the theory derived by Pardee (1951). The data are shown in the legend to the figure.

RESULTS AND DISCUSSION

When a mixture of the ovalbumin hydrolysate and one of the amino acid esters was incubated in the presence of papain, it was observed that the amino acid ester in the reaction system generally decreased in quantity without undergoing hydrolysis to free amino acid to any appreciable extent. Figure 1 shows the time-course decreases of ethyl esters of several straight-chain amino acids as typical examples. Each time-course curve appeared to be linear within 30 min, levelling off after 2 h from the beginning of the incubation. Other L-amino acid esters also showed an almost similar tendency of decrease in quantity, when incubated likewise in the presence of papain. On the other hand, when each amino acid ester alone was incubated in the presence of papain under the conditions described before, neither free amino acid nor polymerization product occurred to any significant extent within 6 h of the incubation period. This indicates that both the ester hydrolysis and the polymerization have only a negligible contribution, if any, to the decrease of any amino acid ester during the plastein reaction.

Table I. Amino Acid Compositions of the Substrate (Ovalbumin Hydrolysate) and of the Plastein Products from this Substrate Mixed with Ethyl Esters of L-α-Aminobutyric Acid, L-Norvaline and L-Norleucine

Amino acid	Weight-percent composition			
	S[a]	A[b]	V[b]	L[b]
Lysine	5.20	5.01	5.06	5.03
Histidine	2.01	1.93	1.90	1.97
Arginine	4.70	4.51	4.63	4.58
Aspartic acid	8.39	8.02	8.03	8.15
Threonine	3.68	3.54	3.48	3.50
Serine	5.72	5.69	5.60	5.61
Glutamic acid	9.33	8.88	8.81	8.79
Proline	1.70	1.65	1.64	1.70
Glycine	3.71	3.71	3.66	3.68
Alanine	5.05	5.12	5.04	5.13
α-Aminobutyric acid		0.41		
Methionine	3.87	3.99	4.01	3.86
Half-cystine	2.50	2.44	2.37	2.40
Valine	8.50	8.83	8.90	8.95
Norvaline			0.59	
Isoleucine	7.18	7.05	6.93	7.10
Leucine	11.76	12.53	12.68	12.47
Norleucine				0.67
Tyrosine	4.89	5.00	5.05	5.06
Phenylalanine	7.01	7.32	7.08	7.13
Tryptophan	1.99	2.02	2.09	2.14

[a] S: substrate. [b] A, V, and L: plastein products from the substrates mixed with L-α-aminobutyric acid ethyl ester, L-norvaline ethyl ester, and L-norleucine ethyl ester, respectively.

A time course of the plastein formation is also shown in Figure 1. Thus a plastein fraction having a molecular weight in excess of 500 was obtained by the Amicon membrane filtration of a 2-h reaction product from the ovalbumin hydrolysate mixed with ethyl ester of L-α-aminobutyric acid, L-norvalline, or L-norleucine, because these unusual amino acids could be conveniently detected and quantified in each plastein fraction. Table I shows the amino acid compositions of these plasteins compared to those of their substrates, indicating that the free amino acids have transferred to the respective plasteins. In particular, the data on the amounts of L-α-aminobutyric acid, L-norvaline, and L-norleucine in the plasteins can reflect their decreases at the incubation time of 2 h (Figure 1).

From all the results obtained above, as well as from those reported in several previous papers (Yamashita et al., 1971; Yamashita et al., 1972; Arai et al., 1974), it is concluded that the decrease of any amino acid ester results from its incorporation with formation of peptide bond.

A series of α-amino acid esters were measured for their reactivities at the incubation time of 30 min, since this

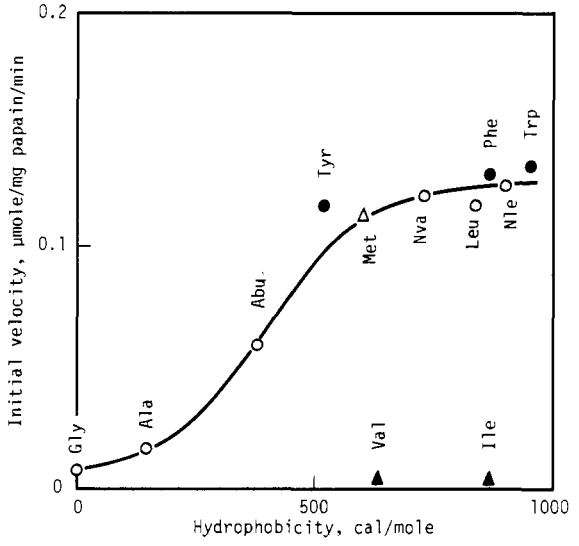

Figure 2. Relationship between hydrophobicities of amino acid side chains and initial velocities of incorporation of amino acid ethyl esters. Symbols for the unusual amino acids: Abu, α-aminobutyric acid; Nva, norvaline; and Nle, norleucine. Data on the relative hydrophobicities: Ala, 159; Abu, 378; Met, 598; Val, 640; Nva, 733; Leu, 838; Ile, 865; Nle, 897; Tyr, 513; Phe, 867; and Trp, 958 (cal/mol).

Table II. Initial Velocities of Incorporation by Papain of L-Alanine and Related Amino Acids Esterified with Straight-Chain Alcohols[a]

Amino acid moiety	Alcohol moiety			
	Ethanol	1-Butanol	1-Hexanol	1-Octanol
L-Alanine	0.016	0.054	0.133	0.135
D-Alanine	0.0		0.0	
α-Methylalanine	0.0		0.0	

[a] Data given as μmol (mg of papain)$^{-1}$ min^{-1}. Each is an average value from duplicated experiments.

could be considered to permit the determination of the initial velocities of incorporation. In each case the measurement was duplicated to obtain a mean value. Figure 2 attempts to plot the initial velocities observed for the amino acid ethyl esters against the relative hydrophobicities of the corresponding amino acid side chains, with the result that; except for the cases of the β branced-chain amino acids, valine and isoleucine (filled triangles), there is a close relationship between the hydrophobicity and the initial velocity, no matter how many different types of neutral α-amino acids are tested including aliphatic (open circles), aromatic (filled circles), and sulfur-containing (open triangle) amino acids.

Another experiment was carried out to investigate the effect of alcohol chain length on the initial velocity of incorporation of L-alanine. It was observed that L-alanine esterified with a more hydrophobic, longer-chain alcohol, i.e., 1-butanol, 1-hexanol, or 1-octanol, was more reactive than that esterified with ethanol (Table II). The data also show that the initial velocity tends to increase as the alcohol chain is elongated. D-Alanine and the unnatural amino acid, α-methylalanine, also were used for comparison, which, however, were found unreactive even if esterified either with ethanol or with 1-hexanol (Table II).

The observed superiority of longer-chain alcohols to ethanol was further confirmed by comparing the reactivities of *n*-hexyl esters of various aliphatic amino acids with those of their ethyl esters (Table III). Especially in the cases of glycine and L-alanine it was found characteristic that their esterification by 1-hexanol was approximately eight–nine times as effective as that by ethanol to improve the initial velocity of incorporation. Even the β branched-chain amino acids were improved for their incorporation, when esterified with 1-hexanol instead of ethanol (Table III).

Table III. Initial Velocities of Incorporation by Papain of Ethyl and *n*-Hexyl Esters of Aliphatic Amino Acids[a]

Amino acid moiety	Alcohol moiety	
	Ethanol	1-Hexanol
Glycine	0.007	0.053
L-Alanine	0.016	0.133
L-α-Aminobutyric acid	0.058	0.132
L-Valine	0.005	0.077
L-Norvaline	0.122	0.151
L-Leucine	0.119	0.140
L-Norleucine	0.125	0.149
L-Isoleucine	0.005	0.048

[a] Data given as μmol (mg of papain)$^{-1}$ min^{-1}. Each is an average value from duplicated experiments.

Table IV. Initial Velocities of Incorporation by Papain of Glycine and L-Norvaline Esterified with 1-Pentanol and Related Branched-Chain Alcohols[a]

Amino acid moiety	Alcohol moiety				
	1-Pentanol	Isopentanol[b]	2-Methyl-1-butanol[c]	*sec*-Pentyl alcohol[d]	*tert*-Butyl alcohol
Glycine	0.054	0.057	0.049	0.048	0.001
L-Norvaline	0.144	0.122	0.132	0.138	0.135

[a] Data given as μmol (mg of papain)$^{-1}$ min^{-1}. Each is an average value from duplicated experiment. [b] γ-Methylbutanol. [c] β-Methylbutanol. [d] α-Methylbutanol.

Next, the effects of different alcohol-chain structures were investigated; 1-pentanol and its stereoisomers having α-, β-, and γ-branches were used whose structures resembled those of the side chains of norleucine, norvaline, isoleucine, and leucine, respectively. Because the α-dimethyl alcohol with the same carbon number was unavailable, *tert*-butyl alcohol was used instead whose structure was analogous to that of α-methylalanine. As the amino acids to be esterified with these alcohols, glycine and L-norvaline were selected which had extremely low and high reactivities, respectively (Table III). These amino acids were esterified with the above five alcohols and each of the resulting ten amino acid esters was subjected to the incorporation reaction. This experiment gave an expected result that the initial velocity of glycine *n*-pentyl ester was distinctly higher than that of glycine ethyl ester (Table IV). Similarly, high velocities were observed also for α-, β-, and γ-methylbutyl esters (i.e., *sec*-pentyl, 2-methyl-1-butyl, and isopentyl esters, respectively) of glycine. Almost similar results were obtained with the same kinds of L-norvaline esters (Table IV). However, an extremely great difference was found between glycine *tert*-butyl ester and L-norleucine *tert*-butyl ester; the former was almost completely unreactive whereas the latter showed a very high reactivity resembling those of others.

To put together the data obtained throughout the study, it is concluded that the initial velocities of incorporation of L-α-amino acid esters are specifically different depending on their structures. In particular, the amino acid side chain structure seems to be of primary importance for any particular amino acid ester to be incorporated effectively (Figure 2). On the other hand, the alcohol chain structure can make significant contribution only when the amino acid moiety is either poor hydrophobic as in glycine

and alanine or sterically unfavorable as in valine and isoleucine (Table III). However, an exceptional case was found in unnatural amino acids such as D-alanine and α-methylalanine where even their *n*-hexyl esters were unreactive (Table II). It should be noted that the structure of the alcohol moiety has a less specific effect than that of the amino acid moiety. For example, L-norvaline, which is sufficiently hydrophobic (Figure 2) and has no β branch, can be effectively incorporated, either when esterified with a poor-hydrophobic alcohol such as ethanol or when esterified with any of the branched-chain alcohols including 2-methyl-1-butanol (analogous to isoleucine) and *tert*-butyl alcohol (analogous to α-methylalanine) (Table IV). In short, the amino acid side chain and the alcohol chain of any particular L-α-amino acid ester can thus be ranked to be primary and secondary, respectively, in terms of the effect on its initial velocity of incorporation during the plastein reaction carried out in the present study.

Previous papers (Yamashita et al., 1971; Arai et al., 1974) have reported that L-methionine ethyl ester added in a soy protein hydrolysate can be effectively incorporated during the plastein reaction by papain. Yamashita et al. (1972), analyzing the resulting methionine-incorporated plastein for its end groups, have disclosed that the methionine residues are mostly located at the C terminals of the plastein molecules. It might well be expected that, in general, amino acid esters added to protein hydrolysate can be incorporated in a similar manner during the plastein reaction by papain. When an amino acid has eventually been incorporated to the N terminal (not to the C terminal) of a substrate peptide, the ester of this amino acid must first react to form an aminoacyl–papain intermediate with liberation of alcohol. It is, however, generally known that the acyl–papain formation from an amino acid ester is not so easy (Glazer and Smith, 1971). Such an amino acyl–papain intermediate, even if formed, must readily undergo hydrolysis, since its deacylation by the hydrolysis may be predominant over that by aminolysis under the condition that the reaction system contains a very high concentration of water (Tanizawa and Bender, 1974). The deacylation by hydrolysis would give rise to free amino acid but, in fact, no appreciable amount of any free amino acid was detected at an early stage of the plastein reaction under our experimental conditions. Any significant occurrence of the amino acyl–papain formation can thus be left out of consideration and so the most probable reaction proceeding is expected to be the peptidyl–papain formation followed by the degradation by an amino acid ester as nucleophile. If this is true, the observed differences in the incorporation velocities of the amino acid ethyl esters (Figure 2) are ascribable to the differences in the affinities of their amino acid side chains for the S_1' subsite which, as well as several other subsites, has been postulated to occur near the catalytic site of papain (Schechter and Berger, 1967, 1968; Lowe and Yuthavong, 1971). For any particular amino acid esters it may generally be possible to relate its reactivity (Tables II, III, and IV) primarily with the affinity between the amino acid side chain and the S_1' subsite and secondarily with that between the alcohol chain and the S_2' subsite (next to the former) of papain.

A further study should be necessary to obtain more information with this respect, because such basic research will eventually have use in some practical process with the plastein reaction for the amino acid incorporation.

LITERATURE CITED

Arai, S., Aso, K., Yamashita, M., Fujimaki, M., *Cereal Chem.* **51**, 143 (1974).

Arai, S., Yamashita, M., Fujimaki, M., *Cereal Foods World* **20**, 107 (1975).

Arai, S., Yamashita, M., Fujimaki, M., *J. Nutr. Sci. Vitaminol.* **22**, 447 (1976).

Arai, S., Yamashita, M., Noguchi, M., Fujimaki, M., *Agric. Biol. Chem.* **37**, 151 (1973).

Aso, K., Yamashita, M., Arai, S., Fujimaki, M., *Agric. Biol. Chem.* **38**, 679 (1974).

Boissonas, R. A., Guttman, S., Jaquenoud, P.-A., Waller, J.-P., *Helv. Chim. Acta* **39**, 1421 (1956).

Brenner, M., Niederwieser, A., Pataki, G., "Thin-Layer Chromatography", Stahl, E., Ed., Springer-Verlag, New York, N.Y., 1969, p 744.

Cipera, J. D., Nicholls, R. V. V., *Chem. Ind. (London)*, 16 (1955).

Durell, J., Fruton, J. S., *J. Biol. Chem.* **207**, 487 (1954).

Eriksen, S., Fagerson, I. S., *J. Food Sci.* **41**, 490 (1976).

Glazer, A. N., *J. Biol. Chem.* **241**, 3811 (1966).

Glazer, A. N., Smith, E. L., "The Enzymes", Vol. III, Boyer, P. D., Ed., Academic Press, New York, N.Y., 1971, pp 501–546.

Lake, A. W., Lowe, G., *Biochem. J.* **101**, 402 (1966).

Lowe, G., Yuthavong, Y., *Biochem. J.* **124**, 107 (1971).

Mycek, M. L., Fruton, J. S., *J. Biol. Chem.* **226**, 165 (1957).

Pardee, A. B., *J. Biol. Chem.* **190**, 757 (1951).

Schechter, I., Berger, A., *Biochem. Biophys. Res. Commun.* **27**, 157 (1967).

Schechter, I., Berger, A., *Biochem. Biophys. Res. Commun.* **32**, 898 (1968).

Schram, E., Moore, S., Bigwood, E., *Biochem. J.* **57**, 33 (1954).

Spies, J. R., Chambers, D. C., *Anal. Chem.* **20**, 30 (1948).

Sluyterman, L. A. AE., Wijdenes, J., *Biochim. Biophys. Acta* **289**, 194 (1972).

Tanizawa, K., Bender, M. L., *J. Biol. Chem.* **249**, 2130 (1974).

Work, T. S., *J. Mol. Biol.* **10**, 544 (1964).

Yamashita, M., Arai, S., Aso, K., Fujimaki, M., *Agric. Biol. Chem.* **36**, 1353 (1972).

Yamashita, M., Arai, S., Fujimaki, M., *J. Agric. Food Chem.* **24**, 1100 (1976a).

Yamashita, M., Arai, S., Fujimaki, M., *J. Food Sci.* **41**, 1029 (1976b).

Yamashita, M., Arai, S., Kokubo, S., Aso, K., Fujimaki, M., *J. Agric. Food Chem.* **23**, 27 (1975).

Yamashita, M., Arai, S., Matsuyama, J., Gonda, M., Kato, H., Fujimaki, M., *Agric. Biol. Chem.* **34**, 1484 (1970).

Yamashita, M., Arai, S., Tsai, S.-J., Fujimaki, M., *J. Agric. Food Chem.* **19**, 1151 (1971).

Received for review February 28, 1977. Accepted May 13, 1977.