Biochemical Characterization of Soybean Protein Consisting of Different Subunits of Glycinin

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Soybean isogenic lines having different glycinin subunit compositions are now being bred. To compare the characteristics of the genetically improved soybean proteins, total protein and purified glycinin proteins were investigated in this study. The absence of subunit(s) resulted in marked changes in the proportion of glycinin to β-conglycinin. When the pH of the total protein and glycinin solution was changed and NaCl was added to the solution, glycinin consisting of only group I subunits and β-conglycinin are highly soluble compared to the $A_5A_4B_3$ or A_3B_4 subunit at a NaCl concentration lower than 0.05 M at pH 5.5. Ultracentrifuge analysis showed that the glycinin containing all subunits was able to assemble to form the 11S structure. That consisting of group I subunits formed a uniform 7S structure. Decreasing glycinin/β-conglycinin ratios and structural changes caused by a lack of glycinin subunit(s) are thus expected to influence the food-processing properties of soybeans.

Keywords: *Soybean; glycinin; mutant; subunit composition*

INTRODUCTION

Soybean seeds contain proteins in a concentration of 40−45% on a dry-weight basis. Glycinin, a major storage protein, accounts for about 35% of total seed protein. Glycinin consists of six subunits, each made up of an acidic (A) and a basic (B) polypeptide component linked by a single disulfide bond (Staswick *et al*., 1984). Five major subunits have been grouped into two subunit groups based on sequence homology, i.e., group I ($A_{1a}B_2$, $A_{1b}B_{1b}$, and A_2B_{1a}) and group II (A_3B_4 and $A_5A_4B_3$) (Nielsen, 1985).

It is known that about 20% of Japanese soybean varieties lack the $A_5A_4B_3$ subunit (Harada *et al*., 1983). A mutant soybean line lacking group I subunits was induced by γ-ray irradiation (Odanaka and Kaizuma, 1989). Kitamura *et al*. (1993) recently found a wild soybean accession lacking the A_3B_4 subunit. It has become possible to genetically manipulate the glycinin subunit composition (Yagasaki *et al*., 1996). Joint segregation of subunit presence and absence fitted the theoretical ratio for independent inheritance among the three loci controlling group I subunits, the $A_5A_4B_3$ subunit, and the A_3B_4 subunit. From this result, we further classified group II into two subgroups, i.e., IIa ($A_5A_4B_3$) and IIb (A_3B_4).

Tofu (soybean curd) is historically one of Japan's most important foods. Glycinin is considered to play an important role in tofu gel formation. In gelatin, glycinin's functional properties have been compared to those of β-conglycinin, the other major storage protein in seeds; the tofu gel from crude glycinin was much harder than that from crude β-conglycinin (Saio *et al*., 1969). In heat-induced gels, Fukushima (1991) indicated that the IIa subunit was closely related to gel formation rate and transparency, whereas the IIb subunit is related to the gel hardness. Therefore, glycinin subunit compositions are also considered to play important roles in tofu gelation. To clarify how glycinin subunit compositions affect tofu gel formation properties, we are currently breeding soybean isogenic lines having different glycinin subunit compositions. In this paper, we report the biochemical properties of soybean proteins containing glycinins having difference subunit compositions.

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MATERIALS AND METHODS

Materials. A cross was made between cv. Tamahomare having group I and IIb subunits and a breeding line having only the IIa subunit. F_3 seeds derived from the cross were used. All soybeans were grown in a field during the summer of 1995. Seeds were classified into eight phenotypes based on the presence or absence of glycinin subunits by Western blot analysis using anti-A_3 serum based on the method of Hirano (1989).

Tris and Coomassie Brilliant Blue G250 were obtained from Sigma Chemical Co. (St. Louis, MO) and Fulka Chemika-BaioChemika (Buchs, Switzerland), respectively. Other reagents were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan).

Analysis of Glycinin−β-Conglycinin Proportion. Fifty milligrams of meal obtained from F_3 seeds was homogenized with 2 mL of distilled water in a test tube for 3 min and centrifuged (15 000 rpm) followed by adjustment of the protein content of the supernatant to 1% (w/w) with distilled water. The protein content was estimated on the basis of the method of Lowry *et al*. (1951) using bovine serum albumin as a standard protein. Ten microliters of the supernatant was diluted with 80 μL of 0.05 M Tris−hydrochloric acid (Tris-HCl) solution (pH 8.0) containing 0.2% sodium dodecyl sulfate (SDS) and 5 M urea, followed by the addition of 10 μL of 2-mercaptoethanol (2-ME). Then, 10 μL of the sample solution was applied to SDS−polyacrylamide gel electrophoresis (SDS−PAGE) 1 h after 2-ME was added. That is, 10 μg of protein was applied to the gel. The gel was stained in 0.2% Coomassie Brilliant Blue G250 in water−methanol−acetic acid (25:22:3) and destained with water−methanol−acetic acid (15:4:1). The proportion of glycinin and β-conglycinin was estimated by calculating the combined area obtained by densitometry.

Precipitation Behavior of Proteins as a Function of Changes in pH and Ionic Strength. Defatted meals were homogenized in 15-fold volumes of 0.05 M Tris-HCl containing 0.098% sodium hydrogen sulfite (SBS) for 2 h. A total protein extract obtained was used to prepare crude β-conglycinin and

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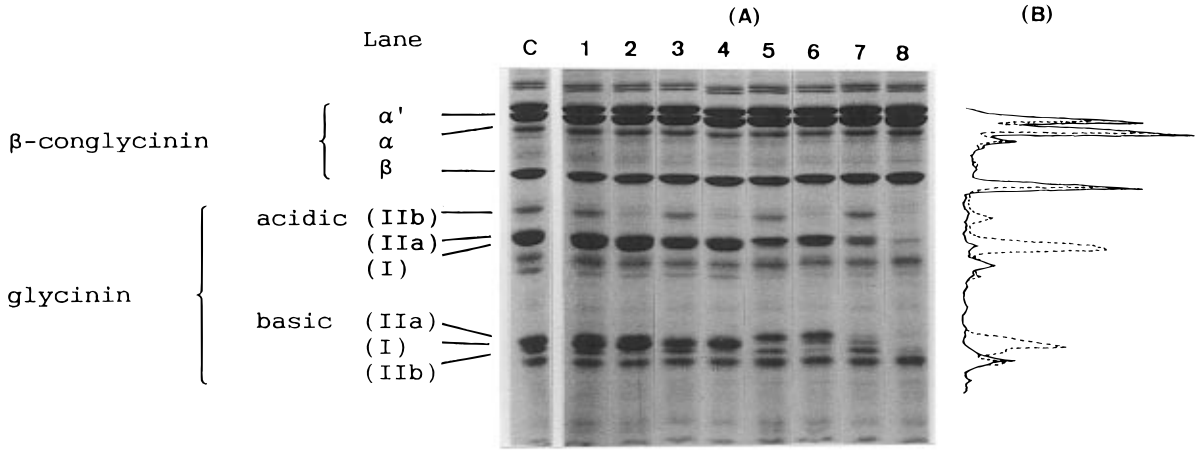

Figure 1. SDS−PAGE patterns of protein obtained from soybean lines having different glycinin subunit compositions (A) and the typical densitometer scanning of the patterns (B): Tamahomare having group I and IIb subunits (lane C); and a soybean line having group I, IIa, and IIb subunits (lane 1 and a dotted line), group I and IIa subunits (lane 2), group I and IIb subunits (lane 3), the IIa and IIb subunits (lane 4); group I subunits (lane 5), the IIa subunit (lane 6), the IIb subunit (lane 7), and lacking three groups (lane 8 and a solid line).

glycinin fractions. The two globulins were isolated according to the method of Nagano *et al.* (1992). Glycinin was further purified on a Concanavalin A Sepharose (Pharmacia Biotech, Uppsala, Sweden) column to remove *β*-conglycinin contamination using a buffer solution containing 0.05 M Tris-HCl (pH 8.0) and 0.098% SBS. Protein samples dissolved in the above buffer were stored at 4 °C and used within a week for further study.

Protein solutions were diluted to 0.02% in 0.03 M Tris-HCl (pH 8.0) containing 0.098% SBS. Turbidities of the protein solutions at different pH values (pH was adjusted from 2.0 to 8.0 using 0.2−1 N HCl solution containing 0.098% SBS) were monitored at 600 nm by spectrophotometer (Hitach U-2000, Hitach Ltd., Tokyo, Japan). The percentage of measured values to the maximum turbidity value was expressed as relative turbidity.

Solutions containing NaCl in different concentrations (up to 0.25 M) were adjusted to pH 5.5 and turbidity was monitored at 600 nm. The percentage of measured values to the maximum turbidity value of the solution without NaCl was expressed as relative turbidity.

Ultracentrifuge Analysis. A Beckman Optima XL-A analytical ultracentrifuge (Spinco Division of Beckman Instruments Inc., Palo Alto, CA) was used. All sedimentation experiments in the present study were carried out in the sedimentation velocity mode at 45 000 or 48 00 rpm and 4 °C. Data obtained were analyzed using the XL-A Data Analysis Program, "Velocity Analysis XLAVEL" (Spinco Division of Beckman Instruments Inc.) to determine sedimentation coefficients.

Sample solutions for the ultracentrifuge analysis were prepared as follows. The crude glycinin fraction was applied to a gel chromatography column of Sephacel S-300 (Pharmacia Biotech) equilibrated and eluted by a buffer solution containing 0.05 M Tris-HCl, 0.5 M NaCl, and 0.098% SBS. The glycinin was further purified on a concanavalin A Sepharose column. The purified glycinin in the buffer with 0.05% sodium azide was stored at 4 °C until use. The purified glycinin solution was subjected to ultracentrifuge analysis after the protein solution was diluted to show an absorbance value of 0.3−0.8 at 280 nm and 1 cm light path with the buffer solution. The buffer was used as the reference solution.

RESULTS AND DISCUSSION

Proportion of Glycinin to *β*-Conglycinin in Seed Extract. To determine the glycinin/*β*-conglycinin (11S/7S) ratio, we used soybean breeding lines having different glycinin subunit compositions. Figure 1 shows the SDS−PAGE patterns of seed extracts obtained from soybean lines and typical densitometric scanning of the patterns. The 11S/7S ratio varied from 1.10 (a line having the three subgroups) to 0.04 (a line lacking the three subgroups) (Table 1). No significant differences were observed in the 11S/7S ratio between Tamahomare and the line having all three groups (row A), between a line having both group I and IIb subunits (row C) and one having group I subunits (row D, where group I subunits mean the three subunits of group I), and between the line having both IIa and IIb subunits (row E) and one having the IIa subunit (row F). The lack of glycinin subunit(s), for instance in row H, generally resulted in a marked decrease of the 11S/7S ratio.

Table 1. Proportion of Glycinin (11S) and *β*-Conglycinin (7S) in Extract

	glycinin subunit composition[a]					
	I	IIa	IIb	7S %	11S %	11S/7S ratio[b]
Enrei	+	−	+	32.2	47.5	1.48[A]
Tamahomare	+	−	+	36.2	40.6	1.12[B]
A	+	+	+	37.6	41.2	1.10[B]
B	+	+	−	39.5	37.9	0.96[C]
C	+	−	+	46.7	30.6	0.66[D]
D	+	−	−	49.5	25.1	0.67[D]
E	−	+	+	46.0	31.0	0.51[E]
F	−	+	−	51.4	24.7	0.48[E]
G	−	−	+	57.7	18.0	0.31[F]
H	−	−	−	71.1	2.8	0.04[G]

[a] + and − indicate presence and absence of the subunits, respectively. [b] Means (two replications) in the same row with different upper case letters are significantly different at the 0.05 level in ANOVA-protected Duncun's multiple range test.

Soybean varieties in Japan can be classified into two phenotypes on the basis of the presence or absence of the IIa subunit in their glycinin. In fact, Taira and Taira (1972) found a wide variation in the 11S/7S ratio among 30 cultivars grown in three locations. However, no report has been made on such low-glycinin varieties such as the lines (rows C and D) described above.

To properly evaluate the tofu-processing suitability of soybeans, it is necessary to determine whether marked changes in the 11S/7S ratio positively or negatively affect the suitability of using total soybean homogenate (soy milk) by using isogenic lines having different subunit compositions of glycinin. As described earlier, the respective glycinin subunits are closely related to gel formation properties in heat-induced gels.

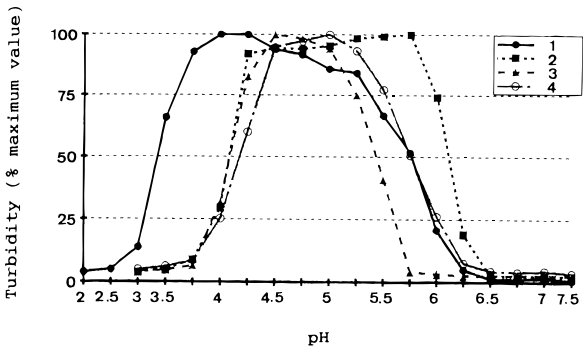

Figure 2. pH-dependent precipitation curves of proteins in 0.06 M Tris-HCl buffer: Total protein (1); crude glycinin (2); crude β-conglycinin (3); and mixture (11S/7S = 1:1) (4). Protein concentration was 0.02% (cv. Fukuyutaka).

Isogenic lines should therefore also be useful in clarifying the relation of subunits to heat-induced gel properties.

Effect of pH and Ionic Strength on the Solubility of Soy Protein Containing Different Glycinin Subunits at about pH 6.0. First, the solubility of soy protein obtained from cv. Fukuyutaka having all glycinin subunits was investigated. Figure 2 shows pH-dependent precipitation curves for total soybean protein, crude β-conglycinin, crude glycinin, and a mixture (1:1) of crude β-conglycinin−crude glycinin solutions with protein concentration adjusted to 0.02% (w/w) with extraction buffer. Crude β-conglycinin behaved differently from glycinin at about pH 5.5: for β-conglycinin the range of maximum insolubility was ~pH 4.25−5.25, whereas for glycinin the range was broader, i.e., pH 4.25−6.0, when the protein pH was lowered with 1 N HCl. Total protein and mixed protein solutions showed precipitation curves between the curves of β-conglycinin and glycinin, suggesting that the turbidity of a 0.02% total protein solution at about pH 6.0 depends on the glycinin−β-conglycinin proportion.

Yamabedaizu, a local variety developed by pure line selection, contains two phenotypes regarding the presence of a glycinin subunit. One has all glycinin subunits (groups I, IIa, and IIb), and the other lacks the IIa subunit. pH-dependent precipitation curves of the two types of glycinins were similar at about pH 6.0 (data not shown). This indicates that the lack of the IIa subunit negligibly affected glycinin solubility.

Figure 3 shows precipitation curves of total protein solutions obtained from breeding lines having different glycinin subunit compositions. Total protein solutions from lines lacking glycinin subunits, except for the line lacking all subunits, showed precipitation curves similar to that of the wild type. The precipitation curve of crude glycinin, which consisted of only one subunit (group I, IIa, or IIb subunit) shifted slightly to the more acidic side compared to that of the total protein. All precipitation curves exhibit essentially maximum insolubility at pH 4.25, which is in the range normally used to make protein isolates. Thus, different subunit compositions have no effect on the solubility in this case. Purified glycinins showed reaction curves similar to that of total proteins (data not shown).

Adding NaCl to total protein solutions in concentrations from 0.1 to 0.25 M decreased turbidity (Figure 4). In contrast, the turbidity of total protein solutions having the IIa subunit increased at 0.05 M NaCl concentration. Changes in the turbidity of total protein, crude glycinin, and purified glycinin solutions containing 0.05 M NaCl at pH 5.5 are shown in Table 2. Because the total protein solution lacking all three glycinin subunits (group I, IIa, and IIb subunits) was turbid, β-conglycinin was presumably responsible for the turbidity of the 0.02% protein solution at that ionic strength (0.05 M NaCl). Crude glycinin was contaminated by the β subunit of β-conglycinin as described by Nagano *et al.* (1992), but crude and purified glycinin showed similar turbidity; that is, the turbidity of glycinin consisting of only group I subunits decreased

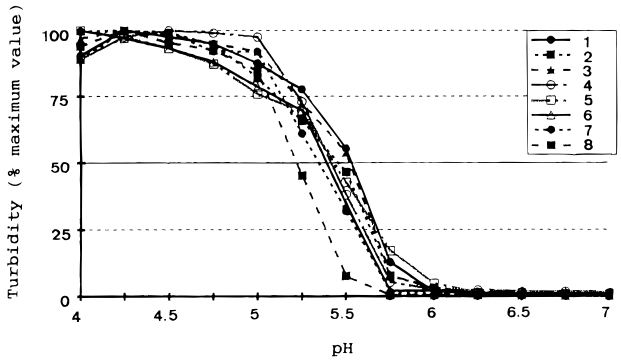

Figure 3. pH-dependent precipitation curves of total protein obtained from breeding lines in 0.06 M Tris-HCl buffer: a breeding soybean line having group I, IIa, and IIb subunits (1), group I and IIa subunits (2), group I and IIb subunits (3), the IIa and IIb subunits (4), group I subunits (5), the IIa subunit (6), the IIb subunit (7), and lacking three subunit groups (8). Protein concentration was 0.02%.

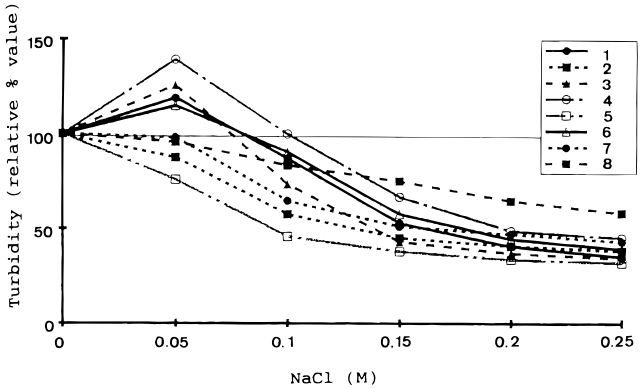

Figure 4. Ionic strength-dependent precipitation curves of total protein in 0.06 M Tris-HCl buffer: a soybean line having group I subunits, IIa and IIb subunits (1), group I and IIa subunits (2), group I and IIb subunits (3), the IIa and IIb subunits (4), group I subunits (5), the IIa subunit (6), the IIb subunit (7), and lacking three subunit groups (8). The protein concentration was 0.02%, pH 5.5.

Table 2. Effect of 0.05 M NaCl on the Turbidity of Total Protein, Crude Glycinin, and Purified Glycinin Containing 0.02% Protein at pH 5.5

glycinin subunit composition[a]			turbidity of total protein		turbidity of crude glycinin		turbidity of purified glycinin	
I	IIa	IIb	0 M NaCl	0.05 M NaCl	0 M NaCl	0.05 M NaCl	0 M NaCl	0.05 M NaCl
+	+	+	100	110[b]	100	162[b]	100	176[b]
+	+	−	100	126	100	157	100	122
+	−	+	100	88	100	125	100	131
+	−	−	100	76	100	95	100	93
−	+	+	100	139	100	104	100	106
−	+	−	100	115	100	106	100	113
−	−	+	100	98	100	147	100	203
−	−	−	100	96	ND[c]	ND	ND	ND

[a] + and − indicate presence and absence of subunits, respectively. [b] Relative value to the value of turbidity of the solution containing no NaCl. [c] Not determined.

Table 3. Sedimentation Species of Various Glycinin Compositions

glycinin subunit composition[a]			sedimentation species and % of glycinin major components
I	IIa	IIb	
+	+	+	10.7S (100)
+	+	−	10.9S (92) and 6.9S (8)
+	−	+	11.0S (63) and 6.3S (37)
+	−	−	10.8S (8) and 6.8S (92)
−	+	+	14.0S (43) and 6.6S (57)
−	+	−	11.0S (51) and 6.4S (49)
−	−	+	12.2S (19) and 6.3S (81)

[a] + and − indicate presence and absence of subunit, respectively.

slightly, while others did not decrease when NaCl was added to 0.05 M (Table 2). Thanh and Shibasaki (1976) have reported that a NaCl concentration up to 0.04 M did not affect the turbidity of 0.02% glycinin in a 0.03 M Tris-HCl buffer (pH 6.0) Our results on glycinin turbidity were consistent with their results, except for that obtained from glycinin consisting only of group I subunits.

These results suggest that group I subunits and β-conglycinin are highly soluble compared to glycinins made of group II subunits (IIa and IIb) at NaCl concentrations lower than 0.05 M at pH 5.5. Thus, both the 11S/7S ratio and the glycinin subunit composition may affect protein solubility at relatively low ionic strengths such as 0.05 M NaCl.

Sedimentation Coefficient of Glycinin Consisting of Different Subunit Compositions. The sedimentation velocity method, one of the ultracentrifuge-based techniques, is considered to be efficient to study biomolecular association (Rivas and Minton, 1996). A recently developed version of an analytical ultracentrifuge (Hensley, 1996) was used to determine sedimentation coefficients.

Table 3 shows sedimentation species observed in glycinins consisting of different subunit compositions. Glycinin consisting of all three groups of subunits and group I and IIb subunits was shown to be able to assemble to form an 11S protein (Figure 5A). Although glycinin having only group I subunits gave a single main peak with a sedimentation coefficient of 7S (Figure 5B), these three glycinins consisting of all three groups of subunits, group I and IIa subunits, and group I subunits, respectively, were assumed to be able to assemble as single uniform structures. In a previous study, we reported that each glycinin subunit can assemble to form glycinin-like proteins, on the basis of gel chromatography analysis (Yagasaki *et al*., 1996), but ultracentrifuge analysis shows that group I subunits assemble to form a structure different from that of the wild type. In contrast, glycinins consisting of both group I and IIb subunits, both group IIa and IIb subunits, and IIa subunit alone gave two peaks (i.e., 11S and 7S) in sedimentation (Figure 5C). Glycinin consisting of the group IIb subunit gave a sedimentation pattern similar to that of glycinin of group I subunits, but the former contained a minor 12S component in addition to a major one. The reason the IIb subunit gave a major peak with a minor one is not yet clear. Mori *et al*. (1982) found reconstitution of 11S and 7S components when the acidic component A_3 or A_4 was mixed with the basic one in sucrose density gradient centrifugation. Our result for the sedimentation coefficient of the IIa and IIb subunits consisting of A_4 and A_3 was consistent with their result.

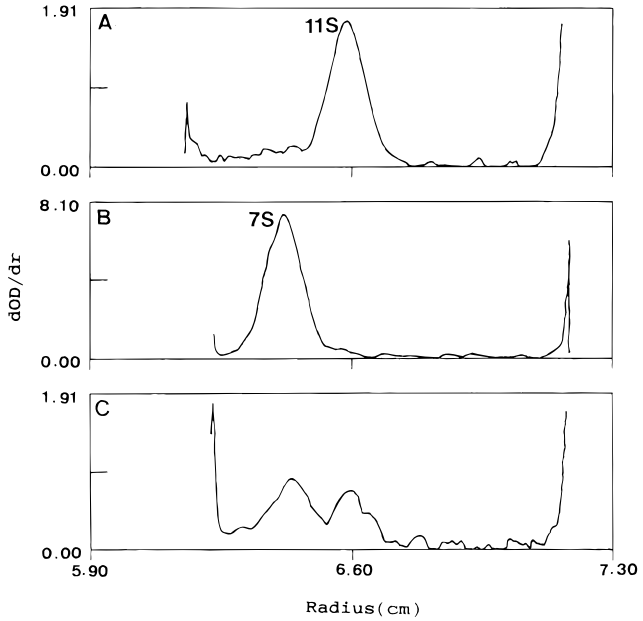

Figure 5. Typical sedimentation patterns obtained at 64 min: glycinin consisting of group I, IIa, and IIb subunits (A), group I subunits (B), and the IIa subunit (C).

Glycinin consisting of both IIa and IIb subunits gave a 14S component in addition to a minor 7S component. Although the 15S fraction was considered to be a minor component of the total extractable protein (Wolf *et al*., 1961), Wolf and Nelsen (1996) found that the 15S component eluted just before glycinin in crude glycinin gel chromatography, and they characterized the component as a glycinin dimer. The 14S component found to be the major fraction in the protein solution of glycinin consisting of IIa and IIb subunits may thus be an assembled form similar to the 15S component.

The functional properties of the soybean proteins must be closely correlated with their assembled state. Further study is thus necessary to more comprehensively characterize the structural aspects of glycinins having different subunit compositions.

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Received for review June 18, 1996. Revised manuscript received November 19, 1996. Accepted November 25, 1996.[⊗]

JF9604394

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1997.