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Effects of Protein Engineering of Canola Procruciferin on Its Physicochemical and Functional Properties

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The primary structure of *Brassica napus* procruciferin 2/3a was engineered to elucidate structure−function relationships and to improve the functionality of cruciferin. The following mutants were constructed: (1) C287T, (2) ΔII, variable region II was deleted; (3) C287T/ΔII, mutation involving (1) and (2); (4) ΔIV + A1aIV; and (5) ΔIV + A3IV, variable region IV was replaced with variable region IV containing many charged residues from soybean glycinin A1aB1b and A3B4 subunits. Differential scanning calorimetry analysis revealed that the A1aIV region has a more favorable interaction with the procruciferin molecule than does A3IV as well as the original regions. On the basis of heat-induced precipitation analysis, it was concluded that replacement of the free cysteine residue with threonine (C287T) and insertion of charged regions (ΔIV + A1aIV and ΔIV + A3IV) could lead procruciferin to form soluble aggregates after heating. Low solubility was observed in mutants ΔIV + A3IV, ΔII, and C287T/ΔII, especially between pH 4 and 6 at μ= 0.08, but not in ΔIV + A1aIV, indicating that the number of acidic amino acid residues and the high number of glutamine residues are important factors for solubility at μ = 0.08. None of the mutants showed any improvements in emulsifying ability, indicating that destabilization and addition of the hydrophilic region are not effective for emulsification. The insertion of the A1aIV region in procruciferin made the molecule more susceptible to α-chymotrypsin.

KEYWORDS: ***Brassica napus*; canola; cruciferin; functional properties; physicochemical properties**

INTRODUCTION

Canola is primarily grown as an oilseed crop. However, canola meal, the major byproduct obtained after seed oil extraction, is rich in protein. The meal contains 36−44% crude protein (*1*). It is considered to be a good protein supplement in the feed trade industry because its protein is of high quality compared to other plant protein sources and is comparable with that of the soybean proteins. For these reasons, it is important to study canola meal proteins and to improve their properties for better utilization either as a feed protein supplement, as a potential ingredient in food systems, or as a component in nonfood or industrial materials. Attempts to improve the functional properties of canola proteins have already been conducted for application in nonfood uses. Most of these involved chemical modifications of the protein such as acylation (*2*), grafting of aliphatic and aromatic groups (*3*), and phosphorylation (*4*). Chemical treatments are not often used in food technology because of reagent toxicity and formation of side-reaction products (*3*). For nutritional quality modifications of canola proteins, antisense technology for both napin (*5*) and cruciferin (*6*) was reported to be promising.

In canola, the major storage protein comprising 60% of the total seed protein is cruciferin (*7*), a member of the 11S globulins. Genomic (*8*−*10*) and cDNA (*11*−*13*) clones of cruciferin have already been isolated and sequenced. There are 9−12 cruciferin genes in *Brassica napus* (*14*) coding for three different precursors, P1, P2, and P3 (*10*, *13*), that give rise to three different mature subunit groups of cru1, cru2/3, and cru4 (*9*). The cru2/3 subunit group consists of two very similar subtypes, cru2 and cru3 (*9*). Each subunit is processed from a single 50 kDa precursor and has a 30 kDa α chain disulfide bonded to a 20 kDa β chain (*15*).

We have previously cloned cruciferin cDNAs cru2/3a and cru2/3b from canola (*16*). Protein expression of these isolated cDNAs was conducted, but we were able to get soluble recombinant protein of only cru2/3a using *Escherichia coli* AD494(DE3) as host. Studies on the application of protein engineering on soybean glycinin (*17*, *18*) and β-conglycinin (*19*) conducted in our laboratory proved that it is an effective method to improve the nutritional, functional, and physiological properties of these globulins. In the present study, we investigated the effects of altering the primary structure of cru2/3a on its physicochemical and functional properties. Site-directed mutations were performed on cru2/3a cDNA, and then the physicochemical and functional properties of the expressed mutant

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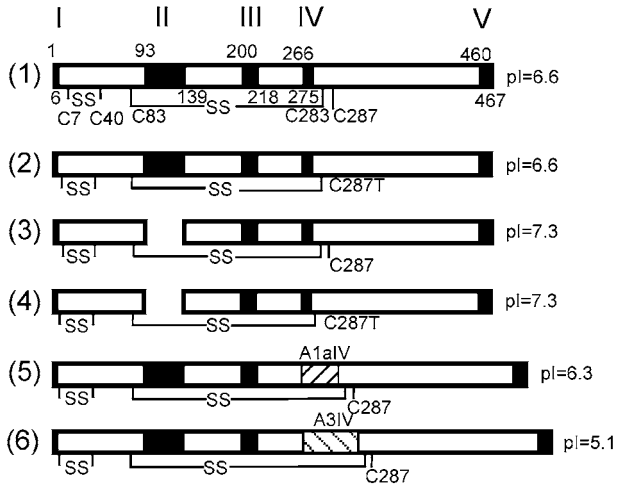

Figure 1. Schematic representation of recombinant procruciferin and mutants: (1) WT; (2) C287T; (3) ΔII; (4) C287T/ΔII; (5) ΔIV + A1aIV; (6) ΔIV + A3IV. Filled and unfilled boxes indicate the variable and conserved regions, respectively, when aligned with other 11S globulins; hatched boxes represent the variable regions IV from glycinin A1aB1b and A3B4 subunits. The calculated p*I* value based on amino acid sequence of each protein is indicated (http://kr.expasy.org/tools/pi_tool.html).

proteins were compared with those of recombinant wild-type (WT) procruciferin cru2/3a and native cruciferin.

It was reported that five of the six disordered regions in the crystal structure of proglycinin correspond to the five variable regions (*20*) identified through alignment of various 11S globulins from legumes and nonlegumes (*21*, *22*). Also, an additional cysteine residue particular to the β chains of the cruciferin subunit was reported (*23*). Here, we made new constructs of cruciferin with modifications on its variable regions II and IV as well as on the additional cysteine residue. Variable region II, the longest variable region of cruciferin, which is rich in glutamine residues (*11*), was deleted, and the shorter variable region IV of cruciferin was replaced with variable region IV, the longest of the glycinin A1aB1b or A3B4 subunit, which is rich in acidic amino acids (*24*). The additional cysteine residue was replaced with threonine.

MATERIALS AND METHODS

Construction of Modified Procruciferins. Cruciferin 2/3a cDNA in pET-21d vector (Novagen) (*16*) was used as a template in site-directed mutagenesis to construct the mutants schematically diagrammed in **Figure 1**. The mutants constructed were (a) C287T, in which Cys287 was substituted with threonine; (b) ΔII, in which variable region II was deleted; (c) C287T/ΔII, in which mutations (a) and (b) were combined; (d) ΔIV + A1aIV and (e) ΔIV+A3IV, in which variable region IV of cruciferin was replaced with the variable region IV of soybean glycinin A1aB1b and A3B4 subunits, respectively. The specific primers and templates used to obtain the constructs mentioned above are given in **Table 1**. Pfu TURBO DNA polymerase (Stratagene) having a high fidelity was used for Polymerase Chain Reaction (PCR). The PCR reaction conditions for mutants C287T and ΔII were as follows: 25 cycles of 20 s at 95 °C, 20 s at 50 °C, 10 min at 68 °C. After PCR, the product was phosphorylated and purified prior to self-ligation. Similarly, the mutation of ΔII was introduced to C287T to construct C287T/ΔII. For ΔIV + A1aIV and ΔIV + A3IV, cDNA regions encoding variable regions IV of A1aB1b (Genbank M36686) and A3B4 (Genbank AB049440) subunits were first amplified using the primers specified in **Table 1**. The amplification was carried out in 45 cycles of 10 s at 96 °C, 10 s at 58 °C, and 10 s at 68 °C. Then, the PCR products were blunted and subsequently phosphorylated. To digest the template, DNA fragments were first treated with *Dpn*I before ligation with the PCR products in which the DNA region encoding variable region IV was previously deleted by PCR reaction involving 30 cycles of 20 s at 95 °C, 30 s at 55 °C, and 10 min at 68 °C.

Table 1. Primers and Templates Used in Modification of Recombinant Procruciferin

mutant	template	primer
C287T	pETCRU2/3	5′-CAGCGCGAGGACCACCGATAACCTC-3′ 5′-GAGGTTATCGGTGGTCCTCGCGCTGC-3′
ΔII	pETCRU2/3	5′GGTGGTATGCACCAGAAAGTGGAGCACATAAG-3′ 5′ACCACCCTCGGCGCATTCAGGGACCACTCTC-3′
ΔIV + A1aIV[a]	pETA1aB1b	5′-CCCACGGACGAGCAGCAACAAAGAC-3′ 5′-TCTTCTGCTTTTGCTTTGGCTTCCTCG-3′
	pETCRU2/3	5′-AACGGTTTAGAAGAGACCATATGCAGCGCG-3′ 5′-CGGCCTAATGACACTGAATGGGCCTTGGAC-3′
ΔIV + A3IV[a]	pETA3B4	5′-AAGTGGCAAGAACAAGAAGACGAAGATG-3′ 5′-TCTAGTCTGACATCCTCTTCCTCCACGTGG-3′
	pETCRU2/3	5′-AACGGTTTAGAAGAGACCATATGCAGCGCG-3′ 5′-CGGCCTAATGACACTGAATGGGCCTTGGAC-3′

[a] Two amplified products by PCR reaction were ligated.

Expression of WT and Modified Procruciferins. *E. coli* AD494(DE3) was previously selected to be the suitable strain for expression of procruciferin (*16*). One milliliter of an overnight culture of AD494(DE3) carrying expression vector for WT or modified procruciferins was inoculated into 500 mL of modified LB medium containing 0.5 M NaCl and 50 μg/mL carbenicillin (eight flasks, 4 L) (*16*). The cells were cultivated at 37 °C with shaking at a speed of 160 rpm using a Sanyo MIR-220 shaker. Four hours later (A_{600} = 0.6), isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 1 mM and further shaking at 25 °C for 20 h was carried out. The cells were harvested by centrifugation at 4800*g* using an RPR-9-2 (Hitachi) rotor in an SCR-60B centrifuge.

Purification of WT and Modified Procruciferins and Native Cruciferin. The cells were resuspended in 35 mM sodium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF), 1.0 μg/mL pepstatin A, and 1.0 μg/mL leupeptin (buffer A). The suspension was sonicated at 300 μA for 15 min with an interval of 30 s for every 30 s of sonication (ultrasonic homogenizer model US-300T, Nihonseiki Ltd.). Cell debris was separated by centrifugation at 4800*g* for 20 min at 4 °C. Initial purification of proteins was done by ammonium sulfate precipitation at 35% saturation for WT, C287T, ΔII, C287T/ΔII, and ΔIV + A3IV and at 45% saturation for mutant ΔIV + A1aIV. The precipitate obtained was dialyzed and applied to an HPLC column for further purification.

WT, C287T, and ΔIV + A1aIV were applied on a Q-Sepharose 26/10 column (LKB Pharmacia, Uppsala, Sweden). For the first two samples, purification was carried out by gradient elution using 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM *p*-APMSF, 1.0 μg/mL leupeptin, and 1.0 μg/mL pepstatin A (buffer B) with an initial NaCl concentration of 0.05 M to a final concentration of 0.3 M after 120 min. For ΔIV + A1aIV, the final concentration of NaCl was 0.5 M. Sephacryl S-300 26/60 (LKB Pharmacia) was employed in purifying ΔII and C287T/ΔII using buffer B containing 0.4 M NaCl. Finally, a Mono-Q column was used to purify ΔIV + A3IV. Gradient elution was conducted using buffer B with initial and final NaCl concentrations of 0.3 and 0.6 M, respectively.

Native cruciferin was extracted and isolated from defatted canola (*B. napus* cv. Westar) meal as described previously (*25*).

Gel Filtration. To assess the self-assembly of the protein samples, gel filtration chromatography using a Hi-Prep 16/60 Sephacryl S-200 HR column (LKB Pharmacia) was conducted. Each protein was eluted with buffer A at a flow rate of 0.5 mL/min. The previously reported void volume of the column and elution volumes of protein standards (*26*) were used as reference.

Protein Measurement and Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis. The amount of protein in the samples was determined using a Protein Assay Rapid

Kit (Wako, Osaka, Japan). Bovine serum albumin was used as a standard. SDS-PAGE was conducted using 11% acrylamide following the method of Laemmli (*27*).

Differential Scanning Calorimetry (DSC) Measurement. DSC measurements were carried out using a Microcal MC-2 ultrasensitive microcalorimeter (Micro Cal Inc., Northampton, MA) following the method previously described (*26*). Measurements were conducted at a protein concentration of 1.5 mg/mL in both high and low ionic strength buffers. The high ionic strength buffer having $\mu = 0.5$ was buffer A plus 0.02% (w/v) NaN_3. The low ionic strength buffer having $\mu = 0.08$, on the other hand, was 10 mM sodium phosphate buffer (pH 7.6) containing 0.05 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 1.0 μg/mL leupeptin, 1.0 μg/mL pepstatin A, and 0.02% (w/v) NaN_3. Thermodynamic parameters were calculated on the basis of Non-2-State with zero ΔCp using the ORIGIN program installed in the computer connected to the microcalorimeter.

Analysis of Heat-Induced Precipitation. The protein samples subjected to heat-induced precipitation analysis were prepared as described in the previous section but without 2-mercaptoethanol. Protein solutions of 1 mg/mL were heated at 70, 80, and 90 °C for 5 min followed by cooling at 4 °C. Precipitate in the solution after heating was subjected to SDS-PAGE, and the band intensities were calculated using a densitometer. Results were reported as a ratio of precipitate to the original amount of protein in the sample.

Dynamic Light Scattering. For particle size analysis of proteins in solution, dynamic light scattering (Nicomp 370 submicron particle sizer) was conducted. Protein solution (4 mg/mL) with $\mu = 0.08$ or 0.5 (see DSC Measurement) was filtered (0.22 μm filter), and 400 μL of sample was analyzed at 25 °C. The average mean diameter of the protein in solution was reported.

Solubility as a Function of pH. Protein solubility as a function of pH was analyzed using the method described previously (*28*). The remaining soluble fraction after incubation of an 0.8 mg/mL protein solution at various pH values in both $\mu = 0.08$ and $\mu = 0.5$ for 18 h at 4 °C was calculated by protein measurement. Solubility is expressed as percent of the remaining soluble fraction.

Analysis of Emulsifying Ability. Emulsifying abilities of the protein samples were studied using the method described previously (*28*). Protein solutions at 0.5 mg/mL concentration in $\mu = 0.08$ and 0.5 were prepared as described under DSC Measurement. Analysis for each sample was conducted several times, and a typical pattern was presented.

α-Chymotrypsin Digestion Analysis. Except for native cruciferin, all of the other protein samples were used in this analysis. The samples were previously dialyzed against 35 mM sodium phosphate buffer (pH 7.6) at room temperature. Five microliters of 0.02 mg/mL α-chymotrypsin was mixed with 45 μL of 0.5 mg/mL protein sample. The mixtures were allowed to react at different times from 1 to 60 min. Five microliters of buffer instead of enzyme solution was used to serve as control. To terminate the digestion, the solution was boiled for 5 min and then immediately dipped and kept in ice water for another 5 min. SDS-PAGE was conducted, and the degree of digestion that has occurred was measured. The two kinetic constants, k_1 and k_2, of α-chymotrypsin digestion were calculated on the basis of kinetic model proposed previously (*29*) using the program KaleidaGraph (HULINKS) in curve fitting.

To determine the major cleavage site of α-chymotrypsin, the N-terminal amino acid sequence of the major digestion product of WT was analyzed by Protein Sequencer Procise 490 (Applied Biosystems).

RESULTS AND DISCUSSION

Expression and Protein Assembly. The modified proteins were expressed in *E. coli* AD494(DE3). The expression level of WT, C287T, ΔII, and C287T/ΔII was estimated to be ~18% of total cell proteins, whereas that of ΔIV + A1aIV and ΔIV + A3IV was ~15%. The expressed proteins were purified using chromatography. Because the mutant C287T/ΔII could not be purified by ion exchange column chromatography, both ΔII and C287T/ΔII were finally purified by gel filtration as mentioned under Materials and Methods. As a result, all mutants were obtained in yields comparable to WT. Furthermore, both WT and modified procruciferins were soluble in salt-containing solutions as described below.

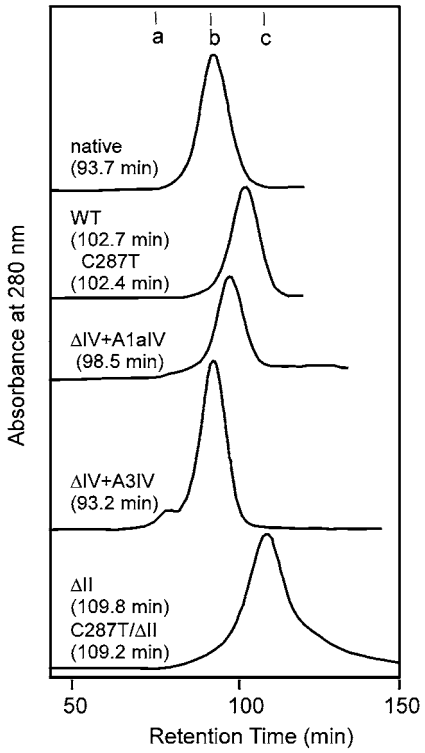

Figure 2. Gel filtration of native cruciferin, WT, and mutants using Sephacryl S-200 HR column. The positions of the void volume (**a**) and the elutions of catalase (240 kDa) (**b**) and bovine serum albumin (67 kDa) (**c**) are indicated.

To examine the molecular assembly of the modified procruciferins, the recombinant proteins and the native cruciferin were subjected to gel filtration chromatography (**Figure 2**). The native cruciferin with a hexameric structure eluted at 93.7 min, whereas WT and C287T eluted at around 102 min, suggesting that the three proteins have a compact structure; also, WT and C287T have a trimeric structure in analogy with the proglycinin molecule (*20*). Deletion mutants ΔII and C287T/ΔII eluted at around 109 min, much slower than WT. This is reasonable because they have lower molecular weights than WT, and accordingly their dimensions became smaller as well. Mutants ΔIV + A1aIV and ΔIV + A3IV, in which the variable regions rich in charged residues were introduced, eluted at 98.5 and 93.2 min, respectively. The latter was close to the elution of the hexameric cruciferin (93.7 min). The replacement of the variable region of the procruciferin with A1aIV and A3IV results in increases of 31 and 59 amino acid residues per subunit. The introduction of such extra residues in procruciferin molecule seems to make the dimension of the trimer larger, probably due to protrusions to the solvent, especially in the case of ΔIV + A3IV. The results obtained here suggest that the recombinant proteins analyzed in this study are able to self-assemble into trimers.

Previously, our group proposed that the criteria for judging the formation of proper conformation of modified proglycinins should be as follows: (1) solubility should be comparable with that of globulins, (2) there must be self-assembly into trimers, and (3) a high level expression must be evident (≥10% of total *E. coli* proteins) (*17*, *18*, *30*). All of the modified procruciferins

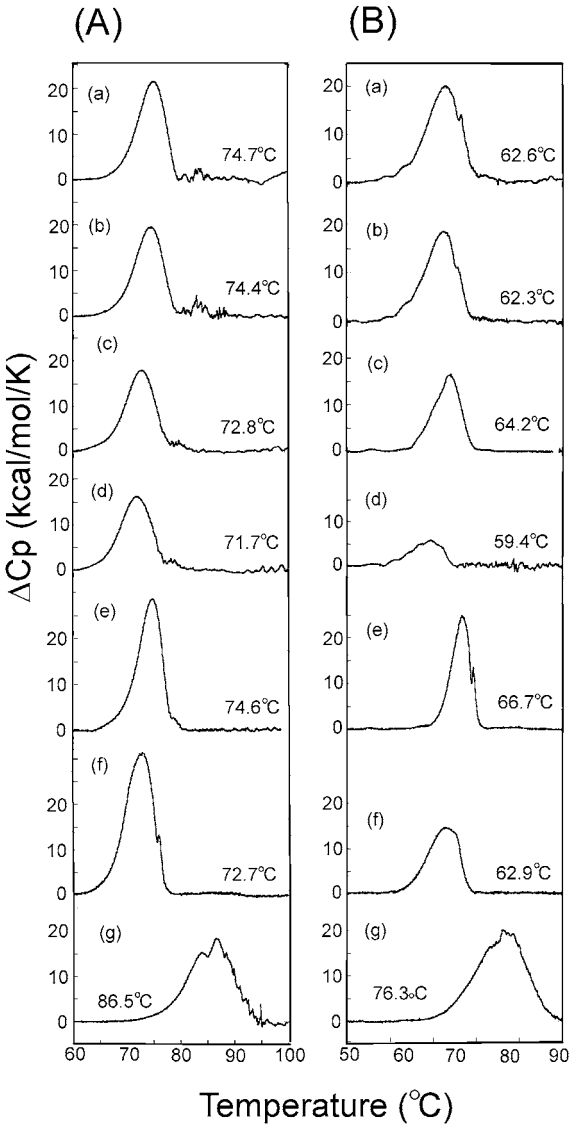

Figure 3. DSC profiles of cruciferin samples at pH 7.6 with (**A**) high ionic strength buffer, $\mu = 0.5$, and (**B**) low ionic strength buffer, $\mu = 0.08$: (**a**) WT; (**b**) C287T; (**c**) ΔII; (**d**) C287T/ΔII; (**e**) ΔIV + A1aIV; (**f**) ΔIV + A3IV; (**g**) native cruciferin. Scan rate was 1 °C/min; protein concentration = 1.5 mg/mL solution.

as well as WT satisfy these criteria, suggesting that the modified procruciferins formed the proper conformation.

DSC Measurement. Structural stability is a factor affecting the emulsifying and gel-forming abilities of a protein and so forth. Here, we studied the structural stabilities of the WT and modified procruciferins and the native cruciferin in terms of thermal properties. Panels **A** and **B** of **Figure 3** show DSC scans of the protein samples, whereas **Table 2** enumerates the thermal denaturation midpoint T_m and enthalpy values (ΔH). Here, the effect of scan rate on the T_m value was tested using three heating rates, 30, 60, and 120 °C/h. It was observed that the T_m value varied only by as much as 1.0 °C among the scanning rates tested. Therefore, application of equilibrium thermodynamics to analyze our data will lead to unfolding parameters close to the true equilibrium values (*31*).

The T_m value of WT was ~11−13 °C lower than that of the native cruciferin at both $\mu = 0.5$ and 0.08. The native cruciferin is composed of three kinds of subunit groups, which associate to form a hexamer molecule, whereas WT is a trimer molecule composed of only cru2/3a subunit. The heterogeneity of the

Table 2. Thermodynamic Parameters Calculated from DSC Experiment[a]

	high ionic strength ($\mu = 0.5$)				low ionic strength ($\mu = 0.08$)			
protein	T_m (°C)	ΔH_{cal} (°C)	ΔH_v (kcal)	$\Delta H_{cal}/\Delta H_v$ (kcal)	T_m (°C)	ΔH_{cal} (°C)	ΔH_v (kcal)	$\Delta H_{cal}/\Delta H_v$ (kcal)
WT	74.7	141	150	0.94	62.6	167	94	1.75
C287T	74.4	133	144	0.92	62.3	167	98	1.70
ΔII	72.8	130	132	0.98	64.2	115	121	0.95
C287T/ΔII	71.7	122	128	0.95	59.4	46	104	0.44
ΔIV + A1aIV	74.6	162	171	0.95	66.7	145	153	0.95
ΔIV + A3IV	72.7	213	145	1.47	62.9	138	100	1.38
native	86.5[b]	ND[c]	ND	ND	76.3[b]	ND	ND	ND

[a] ΔH_{cal} and ΔH_v are calorimetric and van't Hoff heat change, respectively, calculated by curve fitting after concentration normalization using the molecular weight of one subunit; ΔH_{cal} is determined by the transition peak area, whereas ΔH_v is determined by the shape of the transition peak. [b] Temperature at the highest peak of ΔCp. [c] Not determined.

Table 3. Deleted and Inserted Variable Regions

protein	variable region	no. of amino acids	MW	Asp + Glu/ Arg + Lys + His	Gln/Gly
cruciferin 2/3a	II	47 (93–139)[a]	4683	1/1	20/18
cruciferin 2/3a	IV	10 (266–275)	1241	2/2	2/0
glycinin A1aB1b	IV	41 (251–291)	4968	12/11	7/2
glycinin A3B4	IV	69 (252–320)	8295	26/12	8/3

[a] Residue number.

subunit, however, may not adequately explain the higher thermal stability of the native sample. This is because the scan peaks of the native cruciferin and WT were much apart from each other even though the cru2/3a subunit group is the most abundant component among the three subunit groups present in the native sample (*12*). The crystal structure of the glycinin homohexamer showed important residues in electrostatic and hydrophobic interactions, which are conserved for the hexamer formation among the 11S globulin family (*32*). Glycinin was reported to be more stable in hexamer form than in trimer form (proglycinin) (*33*, *34*). Thus, we suggest that the native cruciferin is stabilized by its hexamer structure.

All proteins in this study were less stable at $\mu = 0.08$ on the basis of a comparison of their T_m values between $\mu = 0.5$ and 0.08. This indicates that, in general, unfavorable electrostatic and lower hydrophobic interactions lead to the destabilization of their protein structure at low ionic strength condition. When we compared the mutants with WT individually, the T_m value of ΔIV + A3IV was 2 °C lower than that of WT at $\mu = 0.5$, whereas its T_m value was almost the same as that of WT at $\mu = 0.08$. Probably, charge−charge interactions between the introduced A3IV region and cruciferin molecule are equally favorable and comparable to the case of the original variable region IV. On the basis of calculation by curve fitting, a significantly higher calorimetric enthalpy value (ΔH_{cal}) at $\mu = 0.5$ and a lower value at $\mu = 0.08$ were observed for ΔIV + A3IV than for WT. These results suggest that the introduced A3IV region could be folded rather than randomly coiled at $\mu = 0.5$ and is possibly unfolded at $\mu = 0.08$ due to unfavorable charge interactions in itself because the A3IV region is highly negatively charged (**Table 3**). On the other hand, the T_m value of ΔIV + A1aIV was ~4 °C higher than that of WT at $\mu = 0.08$, and their T_m values were almost the same at $\mu = 0.5$. This result shows that the introduced A1aIV region forms a favorable charge−charge interaction with the procruciferin molecule, resulting in thermal stabilization at $\mu = 0.08$. However, we cannot rule out the possibility of the covering of

hydrophobic residues in the interchain disulfide-containing face (IE-face) (*20*), which may have contributed to the stabilization to some extent. Moreover, the introduced A1aIV region would be folded at $\mu = 0.5$, because the ΔH_{cal} of ΔIV + A1aIV was slightly larger than that of WT at $\mu = 0.5$.

The C287T mutant has T_m values almost similar to those of WT at both $\mu = 0.08$ and 0.5. This result indicates that the replacement of the additional cysteine residue with threonine does not affect the thermal stability of cruciferin. The T_m value of ΔII was 2 °C lower than that of WT at $\mu = 0.5$, whereas at $\mu = 0.08$ its T_m value was 1.6 °C higher. The effect of charge−charge interactions should be low at $\mu = 0.08$, because the deleted variable region ΔII has only two charged residues (**Table 3**). Thus, it is likely that the hydrophobic region of cruciferin becomes exposed due to the deletion of the variable region II and, consequently, makes the molecule unstable at $\mu = 0.5$. Perhaps, the reason ΔII was more stable than WT at $\mu = 0.08$ is due to its decreased conformational entropy at the unfolded state. Interestingly, C287T/ΔII showed significantly lower T_m and ΔH_{cal} values than the rest of the proteins at $\mu = 0.08$, indicating that C287T/ΔII was largely destabilized. On the basis of the crystal structure of proglycinin (*20*), the mutations made are located on the IE-face of the trimer molecule, and the distance between the two mutated sites is only 17 Å. It is possible that the structure around the mutated site was cooperatively changed to an unstable conformation.

Comparison of the directly measured ΔH_{cal} with van't Hoff enthalpy (ΔH_v) provides information on the formation of a cooperative unit during the denaturation process (*35*). A ratio of $\Delta H_{cal}/\Delta H_v > 1$ indicates the presence of folding intermediates during denaturation, whereas a ratio of <1 indicates the formation of an oligomer. In **Table 2**, the $\Delta H_{cal}/\Delta H_v$ ratios of all the proteins except ΔIV + A3IV were almost equal to 1 at $\mu = 0.5$, suggesting that each subunit works as a cooperative unit in the unfolding process. The value of ΔIV + A3IV was significantly >1, indicating the presence of folding intermediate(s). It is likely that the A3IV region is folded separately from procruciferin and its short-range interaction with the procruciferin molecule is weak. The ratio of WT was larger at $\mu = 0.08$ (1.75) than at $\mu = 0.5$ (0.94), implying the presence of intermediate(s) at $\mu = 0.08$, whereas the incorporation of the A1aIV region into the cruciferin molecule makes the $\Delta H_{cal}/\Delta H_v$ value similar between $\mu = 0.08$ and 0.5. This could be due to the fact the A1aIV region works as a cooperative unit with the cruciferin molecule. Eventually, it appears that the variable regions influence the unfolding process at $\mu = 0.08$, and the A1aIV region interacts more favorably than A3IV and other original regions. In addition, the $\Delta H_{cal}/\Delta H_v$ value of ΔII was almost equal to 1 at $\mu = 0.08$, whereas the value of C287T was >1, like WT. In other words, whereas C287T and WT formed intermediates at $\mu = 0.08$, the ΔII molecule behaved cooperatively. This result suggests that the variable region II of cruciferin apparently plays a role in the formation of folding intermediate. The ratio of 0.44 for C287T/ΔII indicates oligomerization (dimer of trimer) at $\mu = 0.08$. This phenomenon was verified by the higher diameter of C287T/ΔII at $\mu = 0.08$ (mean diameter = 9.6 nm) than at $\mu = 0.5$ (mean diameter = 5.2 nm) as determined by a light scattering method; the mean diameter of WT was 6.3 and 6.4 nm at $\mu = 0.08$ and 0.5, respectively.

Analysis of Heat-Induced Precipitation. The gel-forming ability of a protein has many applications in food systems. Good-quality thermally induced gel is formed through the formation of soluble aggregates. Heat-induced precipitation is negatively correlated to heat-induced gelation. The amount of thermally induced precipitates in native cruciferin, recombinant WT, and its mutants at 70, 80, and 90 °C was measured at $\mu = 0.08$ and 0.5.

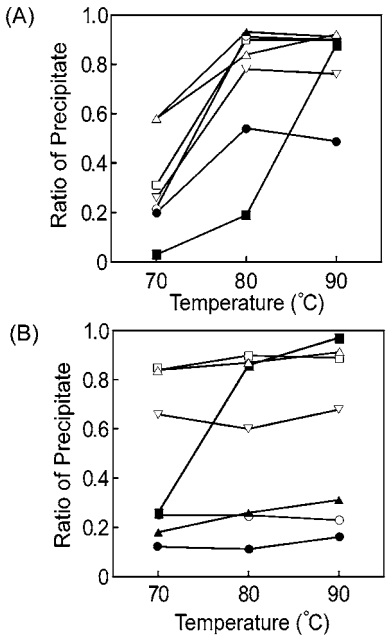

Figure 4. Heat-induced precipitation of cruciferin samples at pH 7.6 with (**A**) high ionic strength buffer, $\mu = 0.5$, and (**B**) low ionic strength buffer, $\mu = 0.08$: (□) WT; (■) native cruciferin; (▽) C287T; (△) ΔII; (▲) C287T/ΔII; (○) ΔIV + A1aIV; (●) ΔIV + A3IV. Protein concentration used was 1 mg/mL.

In **Figure 4** it is shown that there were significant differences between native cruciferin and WT under both conditions. Heating at 70 °C at $\mu = 0.08$ caused precipitation of native cruciferin at a ratio that was ~3 times lower than that of WT, whereas heating at 80 and 90 °C showed no significant difference. This is because at 70 °C, the T_m value of the native sample (76.3 °C) was not yet achieved, whereas the T_m value of WT (62.6 °C) was already reached. At 80 and 90 °C, both proteins were thermally denatured such that no difference in the amount of precipitate formed was observed. Heating at 80 °C and $\mu = 0.5$ caused native cruciferin to produce a much lower ratio of precipitate than WT. The difference is also attributed to their T_m values. Both proteins have a tendency to precipitate upon heating. In the case of native cruciferin, the presence of the additional cysteine is the reason for precipitate formation (*23*). Unfavorable SH/S−S exchange causes the release of basic polypeptides, resulting in precipitation. In the case of WT, the precipitation is probably due to unfavorable hydrophobic association, because no release of the basic polypeptide region occurs in WT because it is in the pro-form.

Comparison between WT and mutants showed three significant differences after heat denaturation at $\mu = 0.5$ (**Figure 4A**). First, C287T was more soluble than WT, although their thermal stabilities based on DSC results were the same. This is probably due to the fact that the SH/S−S exchange reaction with Cys287 causes unfavorable association. Second, ΔIV + A3IV precipitated at lesser amount compared to WT. The A3IV region includes many charged residues, and it appears that the charged region facilitates the formation of less insoluble aggregates. Third, the two deletion mutants formed more insoluble aggregates than WT and all other mutants at 70 °C. This indicates that the deletion at variable region II of cruciferin induces insolubilization by heating.

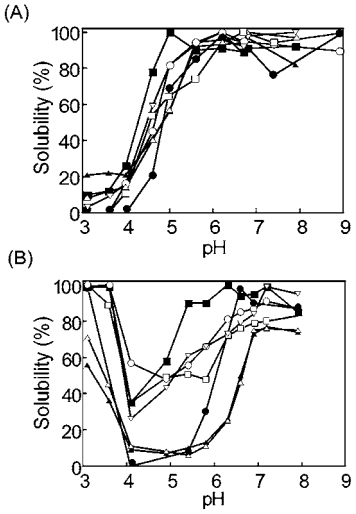

Figure 5. Solubility behavior of cruciferin samples at various pH values with (**A**) high ionic strength buffer, $\mu = 0.5$, and (**B**) low ionic strength buffer, $\mu = 0.08$: (□) WT; (■) native cruciferin; (▽) C287T; (△) ΔII; (▲) C287T/ΔII; (○) ΔIV + A1aIV; (●) ΔIV + A3IV.

At $\mu = 0.08$, a remarkable unprecipitation of C287T/ΔII was observed (**Figure 4B**). According to DSC analysis, C287T/ΔII forms aggregate before heat denaturation on the basis of its $\Delta H_{cal}/\Delta H_v$ value, which is <1. This aggregation was confirmed by light scattering. It is likely that oligomerization before heat denaturation leads to soluble aggregate formation.

Both mutants ΔIV + A1aIV and ΔIV + A3IV were very soluble after heating at $\mu = 0.08$; probably they formed soluble aggregates. The solubility of ΔIV + A3IV aggregates was higher than that of ΔIV + A1aIV at $\mu = 0.5$. It seems that the screening effect of charge interactions at $\mu = 0.5$ inhibits the formation of more soluble aggregates of ΔIV + A1aIV than at $\mu = 0.08$. This is not the case in ΔIV + A3IV, probably due to its higher number of charged residues. Considering the effects of deletion of glutamine-rich variable region II of cruciferin and the insertion of variable regions high in acidic residues from glycinin, we can conclude that charged and hydrophilic regions might lead procruciferin to form soluble aggregates after heat denaturation. However, the effect of charged regions is more effective. These findings suggest that modifications involving highly charged regions as well as removal of free cysteine residue would effectively affect gel formation of cruciferin.

Solubility as a Function of pH. Protein solubility is a fundamental property required for functional properties. We studied the solubility of native cruciferin, recombinant WT, and its mutants at different pH values at both high ($\mu = 0.5$) and low ($\mu = 0.08$) ionic strengths.

At $\mu = 0.5$, all of the cruciferin samples have high solubility at pH >5 and very low solubility at pH <4.5 as shown in **Figure 5A**. The solubility curve of ΔIV + A3IV shifted slightly to the right. The lower solubility of this mutant compared with WT and other mutants at pH 4−5 could be due to a large number of acidic amino acids in the inserted variable region (**Table 3**).

The solubility pattern of the proteins at $\mu = 0.08$ at various pH values was not similar to that at $\mu = 0.5$ (**Figure 5**). The differences in solubility behavior of the proteins can be more easily observed at the low ionic condition because the effects of charge are neutralized by salt ions at the high ionic condition. The solubility was least between pH 4 and 6 at $\mu = 0.09$, whereas it was highest at pH 3−3.5 and pH >7.0. We attribute the decrease in solubility at pH around 4−6 to isoelectric point precipitation (**Figure 1**). The low solubility of mutants ΔII, C287T/ΔII, and ΔIV + A3IV, especially between pH 4 and 6 at $\mu = 0.08$. was more pronounced than that of the others. Also, it can be noted in the figure that the solubility curve at pH 4−6.5 is much wider in these mutants than in the others. The removal of variable region II in cruciferin may have caused the exposure of the hydrophobic region of the molecule in addition to the deletion of many hydrophilic glutamine residues, resulting in greater precipitation than that shown by WT. The difference in solubility behavior of ΔIV + A1aIV and ΔIV + A3IV may be due to the higher content of negatively charged amino acids in A3IV than in A1aIV (**Table 3**).

Table 4. Average Particle Size Distribution Analysis of Emulsions of Procruciferin WT and Its Mutants

	particle size (μm)			
sample	$\mu = 0.5$	SE[a]	$\mu = 0.08$	SE
WT	10.1	2.0	52.3	3.2
C287T	18.5	2.5	56.5	4.6
ΔII	27.4	5.6	90.4	15
C287T/ΔII	29.2	1.8	88.1	2.8
ΔIV + A1aIV	33.9	1.6	49.4	0.6
ΔIV + A3IV	17.9	2.6	93.4	3.6
native	14.2	2.1	17.1	1.2

[a] Standard error.

Analysis of Emulsifying Ability. The emulsifying ability of a protein is one of its significant functional properties in relation to its application in food systems. Proteins which have amphipathic structure with hydrophilic and hydrophobic regions that are polarized in the molecules can exhibit emulsifying ability (*36*). **Table 4** shows the average diameter of particles in emulsions prepared from soybean oil and cruciferin samples. Smaller particle size connotes higher emulsifying ability. The emulsions prepared at the high ionic condition were better than those prepared at the low ionic condition (**Table 4**). Probably, unfavorable charge−charge interaction inhibits the emulsifying abilities of WT and its mutants.

The emulsion particle size of mutants C287T (18.5 μm) and ΔIV + A3IV (17.9 μm) was only slightly larger than those of WT (10.1 μm) and native protein (14.2 μm) at $\mu = 0.5$; mutants ΔII (27.4 μm), C287T/ΔII (29.2 μm), and ΔIV + A1aIV (33.9 μm) exhibited a great decrease in emulsifying ability relative to WT at $\mu = 0.5$. Totally, none of the mutants showed any improvements in emulsifying ability, indicating that destabilization and addition of the hydrophilic region in this study are not effective for emulsification. The variable region II of cruciferin is glutamine-rich (*11*) such that this hydrophilic region could be a contributing factor to the amphipathic structure of the molecule. To understand the decrease in emulsifying ability of ΔIV + A1aIV, further study is needed.

α-Chymotrypsin Digestion. Digestibility of proteins is an important factor affecting their nutritional value. The digestibility of WT and its mutants by α-chymotrypsin was measured. Native cruciferin was not degraded (results not shown), probably because its hexameric structure makes it resistant to α-chymotrypsin.

Figure 6 shows that the degree of digestion increased with time. However, the extent of digestion was least for mutants C287T/ΔII and ΔII, because the major cleavage site of α-chymotrypsin on trimeric procruciferin is within the variable region II based on the N-terminal sequence (QPGGGSP) of the major digestion product, b of **Figure 5A**, of WT. Thus, removal of the protein segment that is primarily attacked by α-chymotrypsin makes the protein more resistant against proteolytic attack. The variable region II is located on the IE-face, which

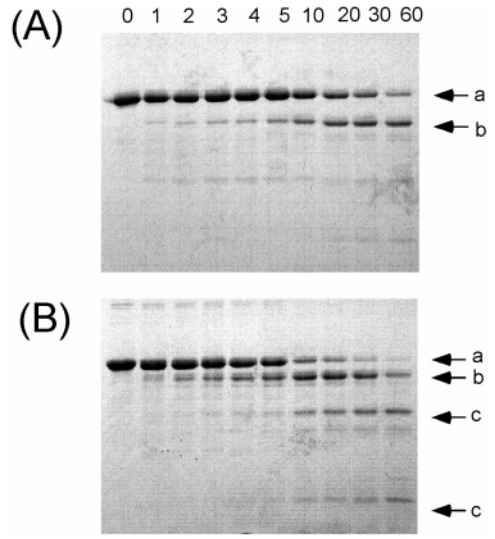

Figure 6. α-Chymotrypsin digestion of WT and mutants: (**A**) WT digestion pattern on SDS-PAGE; (**B**) ΔIV + A1aIV digestion pattern on SDS-PAGE. The numbers indicate the digestion time in minutes. **a**, **b**, and **c** indicate the original protein band, the major digestion product, and the secondary digestion products of **b**, respectively.

Table 5. Kinetic Parameters of α-Chymotrypsin Digestion of Procruciferin WT and Its Mutants

protein	k_1 (%/min)	R [a] (for k_1)	k_2 (%/min)	R (for k_2)
WT	10.1/1.2[b]	0.97	0.42/0.2[b]	0.97
C287T	5.5/1.2	0.90	0.39/0.1	0.99
C287T/ΔII	1.2/0.2	0.49	ND[c]	ND
ΔII	1.3/0.2	0.22	ND	ND
ΔIV + A1aIV	31.6/3.2	0.97	4.33/0.2	0.98
ΔIV + A3IV	8.4/0.4	0.99	1.3/0.1	0.98

[a] Correlation coefficient. [b] Standard error. [c] Not determined

interacts with the same face of another trimer to form a hexamer (*32*); this explains why native cruciferin is resistant to α-chymotrypsin.

Although the deletion of variable region II from cruciferin renders it more resistant to proteolytic attack, the introduction of A1aIV from glycinin subunit makes it more digestible than WT (**Figure 5B**). **Table 5** shows the kinetic constants k_1 and k_2 of α-chymotrypsin digestion. The value of k_1 denotes the rate of disappearance of the original protein per minute, whereas k_2 denotes the rate of disappearance of the major digestion product per minute due to further band cleavage. Note the very low k_1 value of mutants ΔII (1.3) and C287T/ΔII (1.2) and the high k_1 and k_2 values in ΔIV + A1aIV (31.6 and 4.3). The k_1 value of C287T was 2 times as low as that of WT, although the k_2 values were similar between C287T and WT. The size of the major product suggests that α-chymotrypsin attacks WT and ΔIV + A1aIV at the same site, indicating that the introduction of the A1aIV region in procruciferin made the variable region II more susceptible to α-chymotrypsin and that removal of the additional cysteine residue at the β chain made it less susceptible to the enzyme. In other words, deletion of the variable region II and C287 will contribute to less digestibility of procruciferin.

ABBREVIATIONS USED

WT, recombinant wild-type procruciferin; IPTG, isopropyl β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DSC, differential scanning calorimetry.

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