Characteristics of Fraction-1-protein Degradation by Chemical and Enzymatic Treatments

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Crystalline tobacco fraction-1-protein and its large and small subunits were analyzed for polypeptide composition by gradient SDS-PAGE after chemical and enzymatic treatments. Acidic (0.015 N HCl) or alkaline (pH 11) condition at 110 °C cleaved the large subunits of the native protein completely within 1 h, while the degradation of small subunits was negligible. Neutral pH and 110 °C did not cleave the protein. The chemical degradation of individual subunits showed patterns resembling that of the native protein. Immobilized protease, trypsin, papain, and pepsin also cleaved the large subunits of the protein faster than the small subunits; however, the individual subunits degraded in a manner different from that of the protein. Results suggest that the quaternary structure of fraction-1-protein affects the rate and pattern of subunit cleavage by proteolytic enzymes. Although polypeptide composition differed depending upon the treatments, the degradation pattern by a given treatment of fraction-1-protein among *Nicotiana* species appeared to be identical.

Given the increase in world population, protein is identified to be one of the essential and critical nutrients in demand. Among several novel sources of protein, leaf protein has been considered to be the most promising (Milner et al., 1978; Telek and Graham, 1983). Until recent years, the technology of leaf protein isolation has not advanced much past the pioneering work by Pirie (1942). Wildman and his associates (1983) recently developed a simple industrial process to isolate crystalline fraction-1-protein (F-1-p) from young tobacco plants. By the same process, F-1-p from young soybean plants was also obtained in large quantities (Sheen, 1986).

Tobacco F-1-p, which has a balanced composition of amino acids, gave a high protein efficiency ratio (Ershoff et al., 1978) and exhibited exceptional functionality (Sheen and Sheen, 1985). Similar chemical and physical properties have been reported for the F-1-p of soybean (Sheen, 1986) and alfalfa (Knuckles and Kohler, 1982). These findings support the potential use of F-1-p as dietary food. In food formulation and fabrication, proteins require extensive processing and reprocessing before they are in an acceptable form for human consumption. Proteins may thus be subjected to harsh conditions leading to physical and chemical modifications. Tobacco F-1-p is a globular protein of 550 kDa and consists of eight large and eight small subunits of 55 and 12.5 kDa, respectively (Kung, 1976). How the protein is modified in vitro by physical and chemical means could give insight on its stability and degradation pattern and thus provide guidelines with regard to its processing, storage, and usability in the food industry. On the other hand, any treatment leading to a specific cleavage of F-1-p renders academic interest in the phylogenetic evaluation of plant species since the large and small subunits of this protein are independently coded by chloroplast and nuclear genomes (Kung, 1976). The present study examined the degradation pattern of tobacco F-1-p and its subunits when subjected to chemical and enzymatic treatments at various temperatures. The possible application of the degradation pattern on phylogenetic evaluation among *Nicotiana* species was also investigated.

MATERIALS AND METHODS

Preparation of F-1-p and Subunits. Crystalline F-1-p from leaves of tobacco (*Nicotiana tabacum* L.) cultivars Ky 14 and NC 95 representing burley and bright tobacco types, respectively, was prepared by the procedure recently described (Sheen, 1986). To isolate large and small subunits, F-1-p was dissociated in 62.5 mM Tris-HCl buffer, pH 6.8, containing 5% 2-mercaptoethanol and 2.3% sodium dodecyl sulfate (SDS) at 100 °C for 2 min. The protein solution was applied to preparative polyacrylamide gel slabs with a linear gradient of 5–15% acrylamide for SDS-PAGE (Laemmli, 1970). Large and small subunits were visible as clear bands soon after submerging the gel into a 4 M sodium acetate solution. The corresponding gel slices were then collected. The SDS–protein complex was extracted from the polyacrylamide gel with several volumes of the dissociation buffer in the Sorvall homogenizer. After centrifugation, the extract was dialyzed against distilled water in membrane tubing with a cutoff at 6–8 kDa at 4 °C. The retentates were freeze-dried, and subsequent removal of SDS from the protein was achieved by ion-pair extraction according to the method of Henderson et al. (1979).

Chemical Treatments. F-1-p and subunits (0.5–1.0 mg/mL) were treated in acidic solution (0.015 N HCl) by heating at 60 and 110 °C in screw-capped Pyrex culture tubes in a Temp-Block module heater (Rittenhouse and Marcus, 1984). This acidic condition is supposedly specific for cleavage at aspartyl–prolyl bonds. The proteins were also treated in 10 mM glycine–NaOH buffer, pH 11, in a similar manner. Cleavage patterns were monitored with electrophoresis in a time course up to 150 min. Proteins solubilized at neutral pH and subjected to the same temperature treatments were the experimental controls. The experiments were repeated more than three times.

Enzymatic Degradation. Protease (type XVI from *Bacillus subtilis*), trypsin (type III from bovine pancreas), papain (type IV from papaya latex), and pepsin (1:10 000 powder from porcine stomach mucosa) purchased from Sigma Chemical Co. of St. Louis, MO, were immobilized onto porous glass (20–80 mesh) according to the procedure described by Bliss and Hultin (1977). The amount of enzyme bound to porous glass was about 2–3 mg/g of glass as determined by protein solubilization in 1 N NaOH followed by Lowry's protein quantitation (1951). Protein (1 mg/mL) digestion by protease, trypsin, or papain in 0.1 M phosphate buffer, pH 8, and by pepsin in 0.1 M citrate–phosphate buffer, pH 3, was carried out in 50-mL culture flasks in a water bath at 40 °C. The proportion of enzyme-bound porous glass to protein solution was approximately 1:10 (w/v). The flasks were reciprocally shaken at a moderate speed to assure the agitation of the porous glass. Aliquots were taken from the flasks at in-

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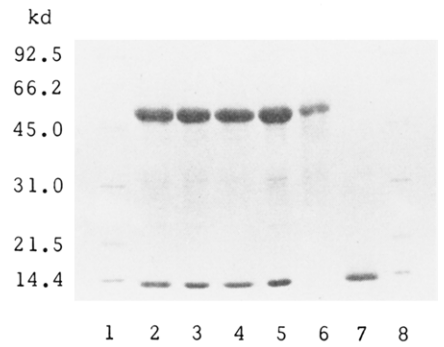

Figure 1. Large and small subunits of F-1-p and individual subunit preparation on the gradient SDS–polyacrylamide gel: lanes 1 and 8, MW markers; lanes 2–5, F-1-p preparations from *N. tabacum* cv. NC 95, Ky 14, *N. sylvestris*, and *N. tomentosiformis*, respectively; lane 6, large-subunit preparation from Ky 14; lane 7, small-subunit preparation from Ky 14.

tervals during the 150-min incubation. The experiments were repeated three times. Proteins incubated with silanized and glutaraldehyde-treated porous glass that had no proteolytic enzymes were the controls.

Polyacrylamide Gel Electrophoresis. Analytical SDS-PAGE by the method of Laemmli (1970) was performed with gel slabs having a linear gradient of 7.5–17.5% acrylamide. Nondenaturing PAGE was also done on 7.5% gel slabs using a discontinuous buffer system (Davis, 1964). Protein samples after the chemical and enzymatic treatments were diluted with the dissociation buffer used earlier to isolate the large and small subunits on the analytical SDS-PAGE and with 0.1 M Tris-HCl buffer, pH 8, for the nondenaturing PAGE. An aliquot containing 30–50 μg of protein from each sample was applied to the gel along with or without molecular weight markers (from BIO-RAD Laboratories). Protein bands were visualized upon staining with Coomassie brilliant blue R.

Phylogenetic Evaluation. In view that the polypeptide composition of F-1-p produced by chemical and enzymatic treatments is highly reproducible and the number of bands appears to be maximal in most cases within 30 min of treatment, the comparison of polypeptide pattern among *Nicotiana* species for a given treatment was therefore based on the 30-min sampling. Leaves from young plants of 23 *Nicotiana* species representing 11 of the 14 taxonomic sections (Smith, 1979) were processed for F-1-p isolation. The purity of F-1-p preparation was affirmed by its subunit composition with SDS-PAGE. Protein isolation, chemical and enzymatic treatments, and SDS-PAGE were performed on the proteins via the aforementioned methods.

RESULTS

Protein Preparation. The freeze-dried crystalline F-1-p was dissociated into large and small subunits of 55 and 12.5 kDa on the gradient SDS–polyacrylamide gel (Figure 1). The isolated subunits appeared at the corresponding sites, thus confirming the high purity of the protein preparation. The freeze-drying process may cause minor degradation of F-1-p as suggested by the appearance of weak bands of about 25 and 33 kDa and occasionally several polypeptides slightly smaller than the large subunit. Similar phenomena were observed with tobacco F-1-p, which has been recrystallized three times by dialysis in a collodion bag (Sheen, 1986). Tobacco cultivars Ky 14 and NC 95 and their progenitor species *Nicotiana sylvestris* and *Nicotiana tomentosiformis* (Gray et al., 1974) were identical in the dissociation pattern of F-1-p. The same results were also obtained for other wild *Nicotiana* species

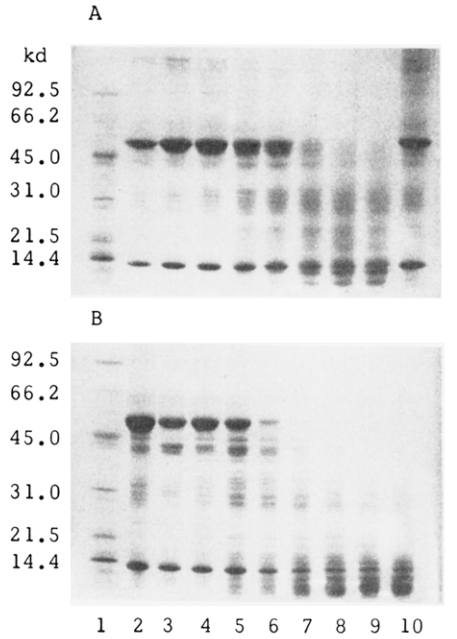

Figure 2. Gradient SDS-PAGE for a time course of F-1-p degradation by chemical treatments at 110 °C. (A) Alkali treatment: lane 1, MW markers; lanes 2–9, sampling times 0, 5, 10, 15, 30, 60, 90, and 120 min, respectively; lane 10, neutral pH and 110 °C for 120 min. (B) Acid treatment: lane 1, MW markers; lanes 2–10, sampling times 0, 5, 10, 15, 30, 60, 90, 120, and 150 min, respectively.

analyzed in the present study.

Chemical Treatments. It is known that alkaline treatment induces hydrolysis of peptide and amide bonds and may also cause cross-linkages of proteins. At pH 11, the degradation of native F-1-p was negligible when incubated for 2 h at 60 °C. With temperature being a function of protein degradation rate, at 110 °C the large subunits of the native F-1-p nearly disappeared within 1 h and became undetectable at the end of 2-h incubation (Figure 2A). In contrast, the same incubation temperature and duration, but in neutral pH, resulted in minimal breakdown of the large subunits. The small subunits degraded slowly under the present experimental conditions. With the exception of two defined polypeptides smaller than the small subunit, the others in the range of 21–33 kDa appeared in smearing. It is worth noting that only at neutral pH the high temperature caused polypeptide aggregation as shown by a smear of proteins larger than the large subunit. This is probably due to heat-induced cross-linking of polypeptides.

Acidic solution (0.015 N HCl) per se slowly broke down the large subunits of the native protein at ambient condition, and the rate of degradation accelerated by elevating the temperature. At 110 °C, the native F-1-p completely lost the large subunits within 1 h (Figure 2B). The disappearance of large subunits was initially accompanied with the appearance of more than 20 smaller polypeptides. As the treatment prolonged, the polypeptides became less in number and smaller in molecular weight. There were at least three polypeptides smaller than the small subunit present in large quantity at the end of 150-min treatment. Similar to the alkali hydrolysis, acidic condition did not degrade small subunits in any significant manner during the first hour, and the degradation rate was very slow thereafter. The degradation pattern of F-1-p from Ky 14 and NC 95 by the chemical treatments was identical and reproducible. When the same samples were analyzed with nondenaturing PAGE, protein smearing appeared throughout the gel. With time, smearing intensity became

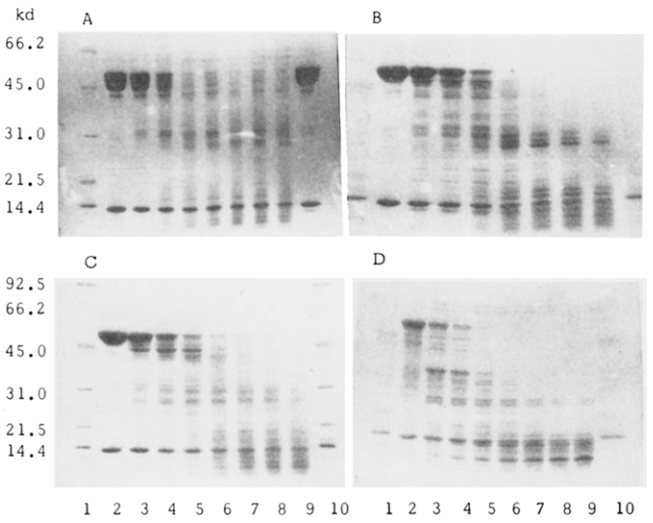

Figure 3. Gradient SDS-PAGE for a time course of F-1-p degradation by immobilized enzymes at 40 °C. (A) Papain digestion: lane 1, MW markers; lanes 2–9, sampling times 0, 5, 15, 30, 60, 90, 120, and 150 min; lane 10, 150-min treatment with porous glass devoid of enzymes. (B) Protease digestion, (C) trypsin digestion, and (D) pepin digestion: lanes 1 and 10, MW markers; lanes 2–9, sampling times 0, 5, 15, 30, 60, 90, 120, and 150 min, respectively.

less pronounced for the samples as a result of continuous breakdown of polypeptides.

Enzymatic Degradation. The degradation of native F-1-p by enzymatic treatments was identical for Ky 14 and NC 95, and thus the pattern of Ky 14 is employed herein for illustration. Papain is a sulfhydryl protease that preferentially cleaves hydrophobic amino acid residues on either side of the residue donating the carbonyl group to the sensitive bond. The initial degradation of F-1-p resulted in a cluster of polypeptides between 42 and 55 kDa (Figure 3A). Subsequent degradation produced polypeptides of 32, 28, and 16 kDa. Several protein bands with molecular weights smaller than that of the small subunit appeared in the samples taken after 1 h of treatment while the quantity of small subunits decreased only slightly. The silanized and glutaraldehyde-treated porous glass alone did not modify F-1-p under the present condition.

Protease catalyzes nonspecific endogenous and exogenous cleavage of protein. The degradation of large subunits started with a cluster of polypeptides between 40 and 55 kDa (Figure 3B). After 30-min incubation, more than 30 polypeptides appeared within the range of 10–55 kDa. While the large subunit became undetectable after 90 min, the small subunit remained identifiable even at the end of 150 min of incubation. Similar patterns of F-1-p degradation were obtained with immobilized trypsin (Figure 3C) and pepsin (Figure 3D). Trypsin supposedly catalyzes the hydrolysis of protein at the carbonyl group of the basic amino acids arginine and lysine. The appearance of a prominent polypeptide of about 44 kDa without polypeptides in the 10-kDa range suggests that the initial breakdown of the large subunit in native protein yields peptides and possibly amino acids too small to be detected by the SDS-PAGE employed. Pepsin is known to cleave peptide bonds linking two hydrophobic, preferably aromatic, amino acid residues such as phenylalanine to phenylalanine or tryosine. Upon the addition of immobilized pepsin into F-1-p solution at pH 3, protein gelled within 1 min. The gel subsequently liquified by the time of taking 5-min samples. Some degradation of the large subunits prior to pepsin digestion can be attributed to the acidic condition. A noticeable characteristic from the pepsin digestion is the appearance of a 5-kDa peptide that steadily

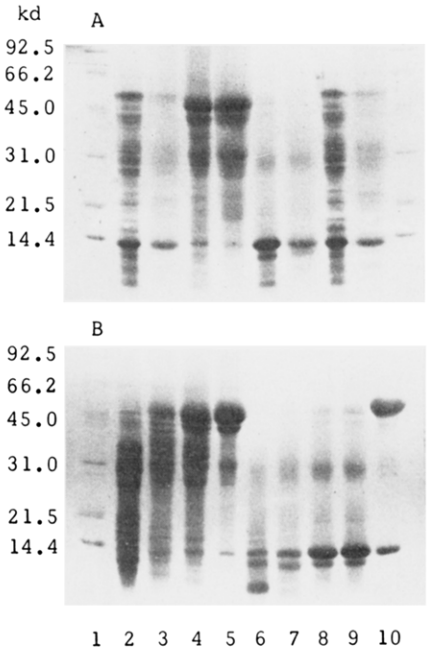

Figure 4. Gradient SDS-PAGE for degradation of individual subunits. (A) Chemical treatments at 110 °C: lanes 1 and 10, MW markers; lanes 2 and 8, 30-min acid treatment for the native F-1-p of Ky 14 and NC 95, respectively; lanes 3 and 9, 60-min alkali treatment for the native F-1-p of Ky 14 and NC 95, respectively; lanes 4 and 5, 15-min treatment of Ky 14 large subunits in the respective acid and alkali conditions; lanes 6 and 7, 15-min treatment of Ky 14 small subunits in the respective acid and alkali conditions. (B) Enzymatic degradation at 40 °C: lane 1, MW markers; lanes 2–5, protease, trypsin, pepsin, and papain digestion of Ky 14 large subunits for 10 min, respectively; lanes 6–9, digestion of Ky 14 small subunits by the respective enzymes for 10 min; lane 10, native F-1-p of Ky 14.

increased in quantity as incubation continued.

Results from the nondenaturing PAGE showed protein smearing without distinctive banding patterns in all samples from the immobilized enzyme treatments. An exception is that of the 5-min samples from the papain and protease treatments, which produced a protein band that moved slightly faster toward the anode than the native F-1-p. It seems to be that the quaternary structure of F-1-p disintegrated rapidly soon after a minor degradation of the large subunits.

Degradation of Individual Subunits. The polypeptide composition of the individual large and small subunits showed a close resemblance to that of the native F-1-p when they were subjected to the acid and alkali treatments (Figure 4A). The treatments degraded the large subunits into a similar banding pattern, and in both cases there was a polypeptide in close vicinity to the small subunit. In addition, acidic degradation resulted in a number of polypeptides smaller than the small subunit, whereas alkaline condition did not yield noticeable bands in that region. Under the treatments, the small subunits appeared to rupture faster in alkaline than in acidic condition. Two weak bands identical in the alkaline hydrolysate of small subunits and native protein suggest that they are of small-subunit origin. Acidic condition coupled with high temperature degraded the small subunits into two major polypeptides of 5 and 10 kDa and into minor ones with intermediate molecular weights. This result also coincides with the acid degradation pattern of native F-1-p. Heat-induced cross-linking of peptides again occurred in both treatments as evidenced by the smearing stain in the region where the molecular weight is greater than the corresponding subunits.

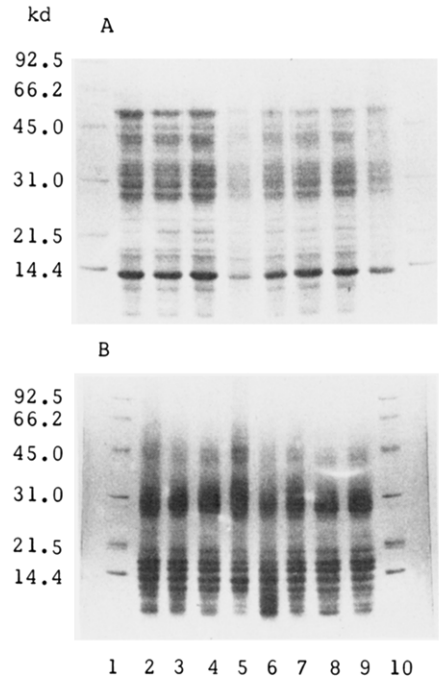

Figure 5. Gradient SDS-PAGE for the polypeptide composition of F-1-p from *Nicotiana* species. (A) Acid treatment for 30 min at 110 °C. (B) Protease digestion for 30 min at 40 °C: lanes 1 and 10, MW markers; lanes 2–9, *N. tabacum* cv. Ky 14, *N. sylvestris*, *N. tomentosiformis*, *Nicotinia glauca*, *Nicotinia rustica*, *Nicotinia bigelovii*, *Nicotinia nudicaulis*, and *Nicotinia rotundifolia*, respectively.

A 30-min digestion of the individual subunits with immobilized enzymes produced a few polypeptides of small molecular weight and with weak staining, suggesting that the subunits alone are more susceptible to enzymatic degradation. In order to examine the degradation pattern, the treatment duration was shortened to 10 min. The polypeptide composition of the enzymatic hydrolysates consisted of a cluster of 28–40-kDa polypeptides (Figure 4B). This contrasts to the cluster of 25–35-kDa polypeptides generated from the degradation of native protein (Figure 3B–D). Although the hydrolysates of the large subunits from the four proteolytic enzymes differed in polypeptide composition, they all had a polypeptide resembling the small subunit in molecular weight. In digestion of small subunits, protease yielded two distinctive polypeptides, whereas the other enzymes produced one each. The strong staining intensity of these bands was not observed in the enzymatic hydrolysates of native protein (Figure 3). Smearing of protein stain, especially in the hydrolysate of small subunits, occurred in all cases.

Phylogenetic Evaluation. Preliminary SDS-PAGE analyses of F-1-p from 23 *Nicotiana* species including *N. tabacum* cultivars Ky 14 and NC 95 by chemical and enzymatic treatments revealed no detectable variation in polypeptide composition. For illustration, seven wild species including the progenitors of modern tobacco and representing all three subgenera as well as a broad geographic origin (Smith, 1979) were compared with Ky 14 for polypeptide composition derived from acid (Figure 5A) and protease (Figure 5B) treatments. The identicalness in polypeptide composition and in banding intensity distribution confirm their closeness phylogenetically.

DISCUSSION

Protein denaturation begins with the change of its secondary and tertiary structure, which is inducible by physical and chemical agents. Most proteins become denatured to form aggregates when subjected to 50 °C or higher. Tobacco F-1-p in neutral pH remains soluble even at boiling temperature, and yet high temperature does not rupture the subunits. This heat stability, however, does not persist if the protein is exposed to acidic or alkaline condition. The present results indicate that tobacco F-1-p can be processed under high temperature so long as pH extremity is avoided. Furthermore, it appears to be more stable in alkaline than in acidic condition at ambient temperature. The acid treatment (0.015 N HCl) supposedly cleaves aspartyl–prolyl bonds upon heating at 110 °C (Rittenhouse and Marcus, 1984). Amino acid sequences of F-1-p subunits from many plant species, including tobacco, do not contain the aspartyl–prolyl bond (Miziorko and Lorimer, 1983). The nonrandom cleavage of the subunits with discrete polypeptide patterns (Figures 2B and 5A) suggests that peptide bonds other than that linking aspartic acid and proline are hydrolyzed by acid. It would be interesting to compare the functionality of the partially degraded F-1-p with that of the native protein. This could give insight on structure and function relationships as far as the functionality of tobacco leaf protein is concerned.

The quaternary structure of tobacco F-1-p is composed of four large subunits stacked over another layer of four large subunits with eight small subunits possibly arranged peripherally (Eisenberg et al., 1978). The initial enzymatic degradation of the native protein removed small oligopeptides from large subunits and still retained a single band on the nondenaturing gel. This suggests the preservation of the quaternary structure. A similar phenomenon has been reported on soybean F-1-p preparation where proteolytic removal of 3- and 5-kDa peptides from large subunits did not result in a loss of small subunits nor the destruction of the quaternary structure of the protein (Paech and Dybing, 1986). Without the quaternary structure, large subunits degraded rapidly by the chemical and enzymatic treatments and produced different polypeptide compositions (Figure 4). In contrast, small subunits seem to be tolerant to the same treatments. This is in agreement with Dehlinger and Schimke's (1970) suggestion that large proteins are degraded faster than smaller ones and dissociation of multimeric proteins would lead to rapid proteolysis of the subunits. Enzymatic digestion of small subunits produced large molecular weight substances that unlikely are a result of peptide crosslinking induced at 40 °C. Enzymatic protein resynthesis, known as plastein reaction, could condense an oligopeptide mixture into high molecular weight polypeptides (Fujimaki et al., 1977). Whether a plastein reaction has been taking place in the enzymatic hydrolysates in the present experiments remains unanswered.

The relative tolerance of small subunits to degradation is of interest. F-1-p, as functional ribulose 1,5-bisphosphate carboxylase/oxygenase in chloroplasts, amounts to more than half of the soluble protein in green leaf (Wildman, 1983). This has led to a speculation that F-1-p may play a role as an amino acid reservoir in nitrogen metabolism. This is supported by the fact that the large subunits, which are coded by chloroplast genome and synthesized within chloroplasts (Kung, 1976), can be readily degradable. The tolerance of small subunits to enzymatic degradation especially from sulfhydryl protease, the predominant protease in tobacco (Liu and Sheen, 1984), may be physiologically important. Small subunits are synthesized in the cytosol where proteases prevail and transported into chloroplasts for assembly into the functional enzyme (Dobberstein et al., 1977).

It is interesting to note that the degradation of large subunits produced a polypeptide similar to small subunits in molecular weight (Figure 4). Whether this coincidence bears any physiological or evolutionary significance between the two kinds of subunits is unknown. In the evolutionary sense, the large subunit is believed to be highly conserved. Variation in polypeptide composition of large subunits in the genus *Nicotiana* was detected by isoelectric focusing patterns of carboxymethylated subunits due to charge heterogeneity (Kung, 1976). This heterogeneity is apparently an artifact during the alkylation procedure that precedes isoelectric focusing (O'Connell and Brady, 1982). This points to the large subunits being homogeneous, the product of a single gene. It is consistent with the identical degradation pattern of F-1-p from *Nicotiana* species (Figure 5). Heterogeneity of small subunits due to amino acid substitution has been unequivocally proved in tobacco (Gray et al., 1978). However, the degradation products of small subunits could be too small beyond detection by the present SDS-PAGE.

F-1-p is an abundant source of protein in green vegetation for human nutrition. Vegetative protein such as soy protein isolate has low lysine to arginine ratio (L:A = 0.9) as compared to animal proteins fish (L:A = 1.4), casein (L:A = 1.9), and milk (L:A = 2.4), and a low L:A ratio is correlated with low serum cholesterol and atherosclerotic incidence in experimental animals (Klurfeld and Kritchevsky, 1986). Tobacco and soybean F-1-p's have L:A ratios of 0.95 and 0.80, respectively, and are better than soy protein isolate in their balanced amino acid composition and excellent functionality (Sheen, 1986; Sheen and Sheen, 1985). When ingested as a food ingredient, one can envision the rapid degradation of F-1-p in the stomach because of the acidity and action of pepsin. The resultant protein fragments will be further degraded by proteases present in pancreatic juice and secreted by microflora in the digestive tract. Enzymatic digestion of casein produced peptides, some of which exhibit physiological effects like opioids (Chiba and Yoshikawa, 1986). Opioid activity was also found in gluten hydrolysate (Zioudrou et al., 1979). The present study demonstrated the degradation of F-1-p into low molecular weight peptides of various amino acid composition. F-1-p has probably been consumed as food in the course of human evolution, but whether its oligopeptide derivatives exert any biological activity to aid human growth and development deserves investigation.

Registry No. HCl, 7647-01-0; NaOH, 1310-73-2; ribulose diphosphate carboxylase, 9027-23-0; proteinase, 9001-92-7; trypsin, 9002-07-7; papain, 9001-73-4; pepsin, 9001-75-6.

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