Comparison of Chemical and Functional Properties of Soluble Leaf Proteins from Four Plant Species†

Shuh J. Sheen

Department of Plant Pathology, University of Kentucky, Lexington, Kentucky 40546-0091

Fraction 1 protein (F-1-p) and fraction 2 protein (F-2-p) were isolated from leaf extracts of alfalfa, soybean, sugar beet, and tobacco. The protein content of F-1-p varied from 97.3 to 99.5%, but greater variation existed for F-2-p. SDS–PAGE analyses revealed that F-1-ps shared the 56- and 14-kDa subunits, but the bandings differed among F-2-ps. Positive correlations were obtained for amino acid composition within the protein fractions. Leaf proteins were sparsely soluble in acidic conditions, but the solubility index increased toward higher pHs. Their foaming capacity and stability were similar within and among the species. Tobacco F-1-p had higher water absorption and fat binding capacity than its F-2-p; however, this was not evident in other species. The latter is substantiated by comparable emulsion properties. The present study demonstrated that F-1-p from diverse plant species exhibits similar chemical and functional properties, and in terms of functionality F-2-p is as good as F-1-p as a dietary protein.

INTRODUCTION

Green leaves can provide proteins, carbohydrates, lipids, vitamins, and minerals for human nutrition if consumed as food. However, edible leaves are few in variety, and their supply varies seasonally. This stems from the fact that not all plant leaves are edible because they contain antinutrients and toxic chemicals. The latter may have a function of repelling predators from perpetuation of species in the course of evolution. Although plant parts are the major source of carbohydrates and lipids in human daily diet, dietary proteins are mainly of animal origin. The present health-conscious society advocates consuming more plant proteins from beans and grains which are known to be deficient in certain essential amino acids. In contrast, the soluble protein from tobacco and soybean leaves possesses an adequate proportion of all essential amino acids (Sheen, 1986). Given the anticipated increase in world population in the coming century, the shortage of protein for human nutrition is imminent. Leaf protein, being the most abundant protein on earth, can alleviate the supply and demand problem. A comprehensive review on leaf protein technology by Kohler et al. (1978) and a recent book, *Leaf Protein Concentrates* (Telek and Graham, 1983), have stressed the very point.

Of the 20–25% dry matter of leafy crops being protein, about one-fifth is soluble protein and the remainder are cellular and chloroplastic membrane proteins which are insoluble. Soluble leaf protein can be categorized into fraction 1 protein (F-1-p) and fraction 2 protein (F-2-p) (Wildman and Bonner, 1947). The former accumulates inside chloroplasts and is the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase that is responsible for photosynthesis and photorespiration (Kung, 1976), whereas the latter is a composite of remaining soluble proteins. An industrial process is available to isolate F-1-p as crystals or precipitates from green biomass (Wildman, 1983). Subsequent acidification of leaf extract yields F-2-p. These simple processes separate edible proteins from undesirable and harmful leaf chemicals such as polyphenols and/or alkaloids. In addition, the leaf protein process produces animal feeds and other byproducts that enhance the economic feasibility of total biomass utilization in the modern agricultural industry.

The usability of protein as a food ingredient in various food forms depends on functional attributes. F-1-p from tobacco exhibits foaming, emulsifying, and gelling properties that are equal to or better than those of known food proteins (Sheen and Sheen, 1985). Similar results were obtained from soluble leaf proteins of soybean (Sheen, 1986) and alfalfa (Knuckles and Kohler, 1982). Recent studies showed that tobacco crystalline F-1-p remains soluble during prolonged boiling (Sheen, 1989). This unusual physicochemical property was not observed in other food proteins. In considering the use of soluble leaf protein from leafy crops as human food, one may ask how F-1-p and F-2-p differ in amino acid composition within and among plant species from the viewpoint of human nutrition and what physicochemical properties in terms of functionality they possess that are necessary in formulated foods. The present study was therefore initiated to answer these questions.

MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* L.), soybean (*Glycine max* L.), sugar beet (*Beta vulgares* L.), and tobacco (*Nicotiana tabacum* L.) were chosen for comparison. Alfalfa leafy materials were separately taken from three field plots on the University of Kentucky Agricultural Experiment Station Farm in Lexington, KY, in the spring when plants were at the flower-bud stage. On the same farm, leaves were harvested from young plants of soybean cultivar Cumberland and tobacco cultivar Kentucky 14 in a manner that three two-row samples constitute three replications. Sugar beet leaves were shipped from Colorado at harvest time. Three shipments representing different harvest dates were processed separately. Harvested leaves were kept in plastic bags at 5 °C until processing. Prior experiments assured that at least in the case of tobacco the storage conditions do not affect the yield and quality of soluble leaf protein in a 1-week period.

Leaf Protein Extraction. Leaves were cut into small pieces, if necessary, and homogenized in a commercial Waring blender

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with a volume of ice-cold 0.5% sodium metabisulfite solution equivalent to 50–75% of fresh leaf weight. Leaf extract was collected by a mechanic presser and subsequently subjected to 50 °C treatment in a water bath for 10 min followed by centrifugation at 10000*g* and 20 °C for 10 min to remove agglomerated chloroplastic particles. Miller et al. (1975) prepared soluble protein concentrate from alfalfa by low-temperature acid precipitation. Similarly, soluble leaf proteins from the four plant species were isolated as F-1-p and F-2-p by cryocrystallization or precipitation and acid precipitation at 4 °C, respectively. The process of protein isolation has been previously described (Wildman, 1983; Sheen, 1986). Protein crystals and precipitates were washed with several volumes of water prior to solubilization at pH 8 and freeze-drying.

Chemical Analysis. Freeze-dried protein powders were digested in the micro-Kjeldahl apparatus and quantitated for ammonia by Berthelot's reaction using ammonium sulfate as the standard (Bradstreet, 1965). Amino acid compositions of F-1-p and F-2-p were determined by digesting samples with 6 N HCl in a N_2 atmosphere for 24 h at 110 °C. Hydrolysates in 0.1 N HCl were quantified for amino acids with a HPLC equipped with an Isco C_{18}, 5-μm packing column, 4.6 × 250 mm, and pre-column derivatization with phenyl isothiocyanate (PTC). The PTC amino acids including norleucine as internal standard were monitored at 254 nm. Sample preparation and analytical conditions were detailed elsewhere (Isco, Inc., 1988). Tryptophan was quantified by the method of Gaitonde and Dovey (1970).

Electrophoretic patterns of F-1-p and F-2-p from the plant species were compared with SDS–PAGE. The PAGE analysis was carried out on gel slabs with a linear gradient of 7.5–17.5% acrylamide. Electrophoretic procedure including molecular weight markers and band visualization with Coomassie Brilliant Blue R-250 was described in a recent paper (Sheen and Sheen, 1987).

Functional Property. The solubility index of the protein preparations was determined at pH 3, 5, 7.4, and 9 in appropriate buffers and by the Bio-Rad protein assay method as a modified Voutsinas and Nakai's procedure (Sheen and Sheen, 1988). Lawhon and Cater's (1971) method for the determination of foaming capacity and stability was followed, whereas water absorption was according to the procedure of Fleming et al. (1974). The turbidimetric method of Voutsinas and Nakai (1983) was used to determine fat binding capacity. The modified turbidimetric method of Pearce and Kinsella for determination of emulsifying activity index by Li-Chan et al. (1984) was adapted except the measurement of A_{500} was carried out with a Cary 210 spectrophotometer. The emulsifying capacity of the proteins was measured by an oil titration method with a volt–ohm meter as described by Regenstein and Regenstein (1984). The emulsifying capacity was defined as milliliters of oil per gram of protein, where oil weight was converted to volume according to specific gravity of 0.918.

Statistics. The data were subjected to the analysis of variance according to a randomized plot design with three replications. Means of the different measurements were compared with the least significant difference (lsd) at the 1 and 5% levels of probability. Correlation coefficients of the amino acid composition of F-1-p and F-2-p between plant species were calculated. Statistical methods were given by Steel and Torrie (1960).

RESULTS

Protein Isolation. The leaf protein process employed in the present study yielded crystalline F-1-p from tobacco leaf but protein precipitates for the remaining species. Freeze-dried powder was snow white for tobacco F-1-p, which has protein content of 99.5%, whereas F-1-p preparations from other species showed gray to yellow appearance with protein content in the range 97.3–98.4% (Table I). Acid-precipitated F-2-ps, which had beige to yellow color possibly as a result of complex formation with phenolic compounds, varied in protein content from 86.5 to 92.4% (Table II). Both protein fractions were odorless and tasteless. No attempt was made to compare the yield of F-1-p and F-2-p among the species since the leaf samples were collected at different times and locations and cultural conditions varied considerably.

Chemical Analysis. Amino acid compositions of F-1-p and F-2-p are presented in Tables I and II, respectively. It appears that F-1-ps of the diverse plant species possess comparable amino acid composition with some variations. Nevertheless, all F-1-ps have essential amino acid content exceeding the reference levels for daily nutritional requirement recommended by FAO and NAS–NRC (Ershoff et al., 1978). There were variations in the amino acid composition of F-2-p among the species. In comparison to the FAO and NAS–NRC reference patterns, alfalfa F-2-p meets the nutritional requirements for the

Table I. Protein Content and Amino Acid Composition of Fraction 1 Protein from Four Plant Species

content and composition	alfalfa	soybean	sugar beet	tobacco	lsd 0.05	lsd 0.01
protein content,[a] %	97.5	97.3	98.4	99.5		
essential amino acid[b]						
Cys	1.3	3.7	3.2	2.9	0.5	0.7
Met	2.1	2.1	2.3	1.5	ns[c]	ns
His	2.4	3.1	2.2	2.3	ns	ns
Ile	5.2	4.3	4.4	4.5	ns	ns
Leu	9.0	9.1	8.1	9.2	ns	ns
Lys	6.2	5.9	5.5	5.8	ns	ns
Phe	5.6	5.6	3.8	4.3	0.4	0.5
Tyr	4.0	5.3	3.8	4.5	0.4	0.5
Thr	5.6	5.2	5.6	5.4	ns	ns
Try	1.7	2.3	1.5	1.5	ns	ns
Val	6.6	4.8	5.6	7.0	0.4	0.6
nonessential amino acid[b]						
Ala	6.6	6.4	8.1	6.9	0.6	0.8
Arg	6.8	7.3	5.9	6.3	ns	ns
Asp	9.3	8.8	9.8	8.5	ns	ns
Glu	11.1	11.0	12.2	11.4	ns	ns
Gly	5.1	5.2	7.7	9.4	0.3	0.5
Pro	4.5	4.0	4.2	4.8	ns	ns
Ser	4.2	3.3	4.7	3.4	0.3	0.4

[a] N × 6.25; content is on dry basis. [b] The quantity of amino acids is in g/100 g of protein. [c] Not significant.

Table II. Protein Content and Amino Acid Composition of Fraction 2 Protein from Four Plant Species

content and composition	alfalfa	soybean	sugar beet	tobacco	lsd 0.05	lsd 0.01
protein content,[a] %	86.5	89.4	90.6	92.4		
essential amino acid[b]						
Cys	0.8	4.2	0.9	0.7	0.9	1.3
Met	2.2	1.5	1.9	3.8	0.4	0.6
His	2.2	4.2	3.4	4.4	0.6	0.8
Ile	4.9	3.4	3.7	3.7	0.5	0.7
Leu	8.3	7.8	5.7	3.5	0.7	1.0
Lys	5.1	5.2	5.6	3.6	0.7	1.0
Phe	4.7	4.9	4.6	7.5	0.4	0.6
Tyr	4.0	4.2	5.6	8.1	0.7	0.9
Thr	4.2	5.5	5.3	4.3	ns[c]	ns
Try	1.2	1.5	1.3	1.4	ns	ns
Val	5.0	3.6	5.8	9.5	0.5	0.7
nonessential amino acid[b]						
Ala	4.7	4.8	4.9	4.7	ns	ns
Arg	5.8	5.4	7.3	9.3	0.6	0.8
Asp	9.4	10.2	8.3	5.6	0.6	0.8
Glu	10.3	11.1	11.2	8.2	0.8	1.1
Gly	5.2	4.4	6.0	3.2	0.3	0.5
Pro	4.6	3.5	4.8	7.1	0.4	0.6
Ser	4.0	3.8	4.4	3.6	ns	ns

[a] N × 6.25; content is on dry basis. [b] The quantity of amino acids in g/100 g of protein. The underlined value indicates a level below that recommended by FAO and NAS–NRC for daily nutritional requirements. [c] Not significant.

Table III. Correlation Coefficient of the Amino Acid Composition of Soluble Leaf Proteins from Four Plant Species

	alfalfa	soybean	sugar beet
		F-1-p	
soybean	+0.94		
sugar beet	+0.92	+0.90	
tobacco	+0.90	+0.89	+0.95
		F-2-p	
soybean	+0.89		
sugar beet	+0.92	+0.38	
tobacco	+0.47	+0.30	+0.65

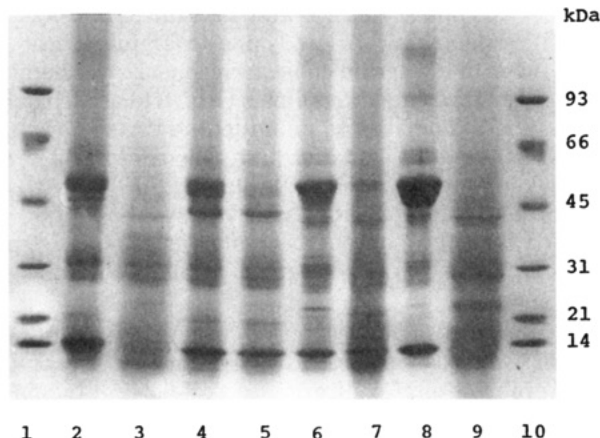

Figure 1. Gradient SDS–PAGE for the soluble leaf proteins of four plant species. Lanes 1 and 10 are molecular weight standards; lanes 2–9 are F-1-p and F-2-p of alfalfa, soybean, sugar beet, and tobacco, respectively.

essential amino acids. Deficiencies include isoleucine and valine for soybean, isoleucine and leucine for sugar beet, and isoleucine, leucine, and lysine for tobacco. A correlation coefficient value as high as +0.95 was obtained between F-1-ps of tobacco and sugar beet, while the lowest value was +0.89 in comparison of tobacco with soybean (Table III). In F-2-p, the value reached as high as +0.92 between alfalfa and sugar beet but only +0.30 between tobacco and soybean. One should bear in mind that F-2-p in cytosol would likely fluctuate during plant growth and development and in response to environmental stresses. The latter could activate different enzymes and in turn alter metabolic processes depending on stress factors. A meaningful comparison of F-2-ps among plant species therefore may not be possible.

SDS–PAGE revealed identical large and small subunits with respective molecular weights of 56 000 and 14 000 for the F-1-ps of the four species (Figure 1). This substantiates the similarity in molecular mass of F-1-ps from plants reported by others (Miziorko and Lorimer, 1983). Several weak bands with molecular weights of less than 56 000 appeared in the F-1-p preparations. This was probably attributable to the freeze-drying process that might cause breakage of the large subunits. Likewise, the dehydration of protein may result in irreversible aggregates that appeared as high molecular bands in the F-1-p and F-2-p preparations. Variation in banding pattern of F-2-ps among the species was evident. There was no detectable bands corresponding to the large subunit of F-1-p in the F-2-p preparations. This ascertains a complete separation of F-1-p from the leaf extract by the present processing procedure. An intense band with molecular weight similar to that of the F-1-p small subunit was present in the F-2-p of soybean and sugar beet. Their identity to the small subunit was not verified. However, the appearance of the small subunit in cytosol is not surprising since it is coded

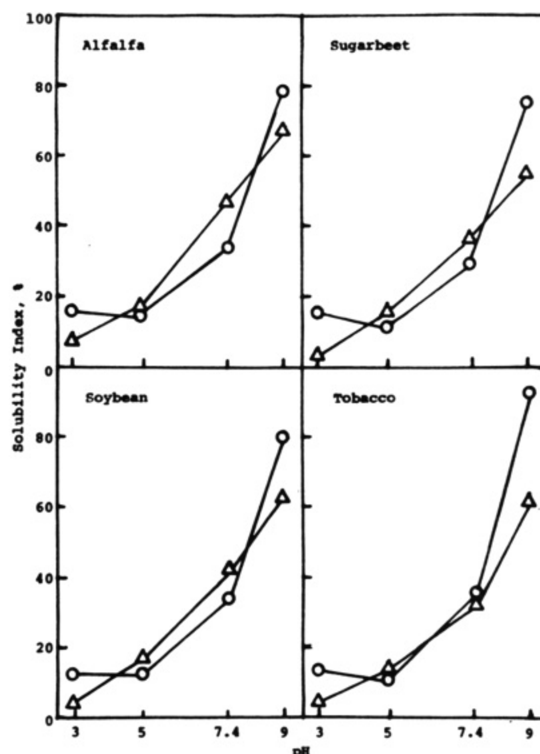

Figure 2. Change of solubility index of F-1-p (O) and F-2-p (Δ) from alfalfa, soybean, sugar beet, and tobacco in 0.1 M each of citrate–phosphate buffer, pH 3 and 5, phosphate buffer, pH 7.4, and boric acid–borax buffer, pH 9.

by nuclear genes and synthesized in cytoplasm prior to transporting into chloroplasts (Kung, 1976).

Functional Property. Although both leaf protein fractions were sparsely soluble at pH 3 and 5, F-1-p was more soluble at pH 3 than F-2-p. At pH 5, the approximate isoelectric point of F-1-p (Bahr et al., 1977), F-2-p was slightly more soluble than F-1-p (Figure 2). The solubility index was increased toward alkalinity for both proteins. At pH 7.4, the solubility index was around 40%. F-1-p increased solubility much more than F-2-p at pH 9, where tobacco F-1-p was greater than 90% soluble and the others were around 80%. At the same pH, F-2-p was less than 70% soluble. Nevertheless, the solubility index patterns of a given protein fraction nearly superimpose one another among the species.

Previous studies comparing egg white, casein, and soy protein isolates for a number of functional properties affirmed the superior functionality of tobacco F-1-p (Sheen and Sheen, 1985, 1988). Thus, tobacco F-1-p serves as a reference in the present study to measure the same functional properties. Foaming capacity was lowest for sugar beet F-1-p, which was, however, not significantly different from its F-2-p (Table IV). This also held true for other species. Leaf proteins had excellent foaming stability. The decrease in foam volume was only 10–15% over 2 h. Even though there was no difference in foam volume between F-1-p and F-2-p, the latter appeared consistently higher in alfalfa, sugar beet, and tobacco. Among the species, sugar beet seems to be slightly inferior in foaming property.

Tobacco F-1-p absorbed more than 4 times its weight in water, while the other protein preparations fell in the range of 3.5-fold (Table V). Tobacco F-1-p also exhibited the highest fat binding capacity, but the others showed no difference in this functionality. This points to the fact that F-2-p is functionally as good as F-1-p in most cases. Similarly, emulsifying properties in terms of emulsion

Table IV. Foaming Capacity and Stability of Soluble Leaf Proteins from Four Plant Species

plant species	leaf protein	foaming capacity,[a] mL/0.5 min	foaming stability,[a] mL			
			10 min	30 min	1 h	2 h
alfalfa	F-1-p	83.7	32.3	30.0	29.0	28.0
	F-2-p	86.0	37.0	35.7	33.7	33.7
soybean	F-1-p	86.0	37.0	34.7	33.7	32.0
	F-2-p	84.7	34.7	32.7	32.0	31.0
sugar beet	F-1-p	77.7	27.0	26.0	25.0	24.7
	F-2-p	80.0	30.3	29.0	27.7	27.3
tobacco	F-1-p	82.0	33.3	30.3	28.7	27.6
	F-2-p	85.7	38.7	35.7	35.3	33.3
lsd	0.05	4.0	4.0	5.9	5.5	4.5
lsd	0.01	5.5	5.5	8.2	7.6	6.2

[a] Determined by the method of Lawhon and Cater (1971).

Table V. Emulsification-Related Properties of Soluble Leaf Proteins from Four Plant Species

plant species	leaf protein	water absorption,[a] %	fat binding capacity,[b] %	emulsion activity index,[c] m^2/g	emulsion capacity,[d] mL of oil/g
alfalfa	F-1-p	346	114	127	431
	F-2-p	347	101	124	430
soybean	F-1-p	339	124	116	423
	F-2-p	327	120	110	433
sugar beet	F-1-p	346	128	107	437
	F-2-p	338	140	132	449
tobacco	F-1-p	424	188	131	453
	F-2-p	334	133	127	426
lsd	0.05	38	40	ns[e]	ns
lsd	0.01	53	56	ns	ns

[a] Determined by the method of Fleming et al. (1974). [b] Voutsines and Nakai (1983). [c] Li-Chan et al. (1984). [d] Regenstein and Regenstein (1984). [e] Not significant.

activity index and emulsion capacity were not found to be different between the protein fractions and among the species (Table V).

DISCUSSION

F-1-p of higher plants is composed of eight large subunits and eight small subunits with molecular weights slightly larger than 500 000 (Kung, 1976). The large subunit is encoded in the chloroplast DNA, while the small subunit is a product of more than one gene within the nuclear DNA. The study of amino acid sequence with the evolutionarily most distant F-1-p polypeptides (*Chlamydomonas* versus *Zea mays*) revealed greater than 85% homology in a total of 475 residues (Miziorko and Lorimer, 1983). Although small subunits were not as highly conserved as large subunits, there was about 70% homology among the sequences of spinach, soybean, and pea small subunits. Amino acid sequence analyses thus substantiate the highly positive correlation in amino acid composition among the species in the present study (Table III). In addition, the similarity of amino acid sequence necessitates the formation of similar oligomeric structure which determines physicochemical property. On the basis of F-1-p crystallization conditions for eight plant species, it has been suggested that the surface charges of this protein are highly conserved (Johal et al., 1980). This may in part explain the resemblance of functional characteristics among the F-1-ps of different species. Variation in the amino acid composition of F-2-p among the species is expected due to species variation as well as different developmental and environmental conditions. However, such variation did not modify the functional properties. Whether this reflects physicochemical features that render solubility in cytosol can only be speculative. Nevertheless, F-2-p can be used as protein supplement in various food systems because of its exceptional functionality.

As to nutrition in terms of the content of essential amino acids, F-1-p is better than F-2-p. F-1-p from tobacco being higher in protein efficiency ratio than casein has been reported (Ershoff et al., 1978). Both F-1-p and F-2-p showed a lysine to arginine ratio of less than one (Tables I and II). In contrast, casein and milk have ratios of 1.9 and 2.4, respectively. A low lysine to arginine ratio is correlated with low serum cholesterol and atherosclerotic incidence in experimental animals (Klurfeld and Kritchevsky, 1986). This can be an added advantage of consuming leaf protein as daily diet. The use of F-1-p as human food was advocated by Wildman (1979) more than a decade ago, and the term "Rubisco", an abbreviation of ribulose 1,5-bisphosphate carboxylate/oxygenase (F-1-p), was coined by his UCLA colleague to parallel with Nabisco, a food company.

The process of green vegetation for edible protein can be attractive if economic return is satisfactory. This may be achieved by total utilization of agricultural biomass through the leaf protein process. In the case of alfalfa, the excess amount of leaf protein in biomass does not enhance the growth and development of grazing animals. The fibrous residue from the leaf protein process is in fact a better feed due to an adequate level of protein and an improved digestibility of the fiber (Kohler et al., 1983). The leaf of sugar beet at harvest is often plowed under as green manure. Again, the leaf protein process can convert the leaves into edible protein for human consumption and fibrous residues as animal feed. Similarly, leaf protein and animal feed can be obtained from young soybean plants (Sheen, 1986). Harvesting young plants as biomass shortens soybean growth duration by more than half of that needed for conventional cultivation so that it can easily fit into any agricultural cropping system. Furthermore, the high concentration of sulfur-containing amino acids in leaf protein can compensate for the sulfur deficiency in seed protein when soybean seed and leaf proteins are used as a mixture. Leaf protein may also improve the functionality of seed protein.

The economic benefits of the leaf protein process are far greater for tobacco. Tobacco F-1-p in crystals is probably the best protein among the existing food proteins for nutrition and functionality, and it may have pharmaceutical usage for renal and postsurgical patients as well. In addition, after selective removal of undesirable leaf constituents, the deproteinized tobacco leaf fiber and leaf juice can be processed into a smoking material that contains less health-harmful substances upon smoking (Wildman and Sheen, 1981). In other words, tobacco offers dietary protein to better the nutritional quality of human diet as well as smoking material to minimize the potential of tobacco smoke and health problems. This is especially important for less developed countries where the smoking population is increasing and human malnutrition is overwhelming.

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