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Susceptibility of Phaseolin to in Vitro Proteolysis Is Highly Variable across Common Bean Varieties (*Phaseolus vulgaris*)

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A study was conducted to investigate the amino acid (AA) composition and the susceptibility to in vitro proteolysis (pepsin, 120 min and pancreatin, 240 min) of a collection of purified phaseolins ($n = 43$) in unheated or heat-treated form. The AA composition of phaseolin varied little across bean varieties. At 360 min of in vitro proteolysis, the degree of hydrolysis varied from 11 to 27% for unheated and from 57 to 96% for heated phaseolins ($P < 0.001$). Heat treatment markedly increased the susceptibility of phaseolin to proteolysis ($P < 0.001$). The AA scores (AAS) and the protein digestibility corrected for AAS indicated S-containing AA as the limiting AA (39 ± 3 and 30 ± 5%, respectively). In conclusion, susceptibility to proteolysis of heat-treated phaseolin rather than its AA composition affects the nutritional value of phaseolin estimated in vitro. Therefore, it should be the criterion of choice in breeding programs aimed at improving the nutritional value of common beans for humans.

KEYWORDS: ***Phaseolus vulgaris*; phaseolin; proteolysis; nutritional value**

INTRODUCTION

Phaseolus vulgaris beans represent an important source of dietary protein for humans in various regions of the world (*1*). As other benefits, it promotes health; this information tends to stimulate bean intake (*2, 3*). The bean storage globulin phaseolin (7S fraction, vicilin family) makes 40−50% of total protein (*4*). Its nutritive value is limited by a low sulfur amino acid (AA) content and a high resistance of the protein to enzymatic hydrolysis (*5, 6*). Heat treatment is known to improve drastically phaseolin hydrolysis (*7, 8*). However, this is not always sufficient to obtain a satisfactory nutritional value.

Many genetic variants of phaseolins have been reported with different numbers (3−6) and molecular weights (MW) of subunits (*9*). Molecular diversity of phaseolin has been used mainly as an evolution indicator of bean domestication in Central America and in the Andes region. It provides solid botanic, archeological, and historical information because of polymorphism, environmental stability, and biochemical complexity characteristics (*10*). The variations in MW and isoelectric point of subunits originate in the DNA sequence (two different phaseolin precursors: α with 435–444 AA and β with 421 AA (*11*)) and in co- and post-translational modifications (*10*).

Little information in terms of AA composition and susceptibility to in vitro or in vivo proteolysis of phaseolin types is available despite the reported high phaseolin diversity. Such investigations are limited to three phaseolins, namely, S, T, and I. Montoya et al. (*12*) showed that the degree of hydrolysis (DH) in vitro of T and I phaseolin was higher than that of S phaseolin. Conversely, one in vivo study reported lower ileal digestibility for T-phaseolin-containing beans as compared to S-phaseolin-containing beans in pigs (*13*). Moreover, differences in methionine content (7.5−10 mg/g protein) have been shown for different phaseolins (*4*).

Plant breeders would like to take advantage of differences in phaseolin subunit and AA composition or hydrolysis potential for developing bean varieties with improved nutritional value. But this will be possible only if the variability in AA composition and in susceptibility of phaseolin to proteolysis are determined.

Therefore, the present work was carried out to investigate the biochemical diversity of 43 phaseolins in terms of (a) AA

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Table 1. Geographical Information and Nutritional Composition of the Different Bean Accessions Used for Phaseolin Purification and in Vitro Hydrolysis

		geographical information[a]			nutritional composition (g/kg DM)[b]		
phaseolin code	CIAT accession no.	origin	province	seed weight (g)[c]	CP	NDF	starch
			Cultivated				
He	G51006	COL	Antioquia	94.6	238	221	393
Ca1	G50850	COL	Antioquia	75.4	231	223	448
Li	G51019	COL	Antioquia	64.2	245	217	317
To2	G23786B	PER	Apurimac	61.0	197	232	400
Ko	G23620	BOL	Cochabamba	50.0	222	197	465
Pa	G23771	PER	Apurimac	50.0	202	219	479
A1	G12078	PER	Ayacucho	47.0	212	255	473
Car	G50412	COL	Antioquia	44.6	247	268	431
To1	G4472	CHI		39.1	250	187	344
Ti2	G51036	COL	Cauca	35.8	255	313	356
H1	G51288	COL	Cauca	35.1	242	177	307
Qui	G24674	COL	Cundinamarca	33.6	211	223	435
C	G24664	COL	Cundinamarca	31.2	207	232	441
Ti1	G51048	COL	Cauca	23.4	225	247	321
S	G51400	COL		19.0	191	246	332
I	G51400	COL		17.0	215	260	319
mean ($n = 16$)				**45.1**	**224**	**232**	**391**
SD				**20.9**	**20**	**33**	**63**
			Wild				
Ch	G50886	COL	Antioquia	21.0	224	291	374
M6	G24665	COL	Cundinamarca	16.0	219	225	368
K	G23422	PER	Apurimac	14.0	243	306	321
L	G24408	COL	Cundinamarca	10.6	233	356	343
J3	G19902	ARG	Tucuman	10.0	278	307	326
P1	G23423	PER	Apurimac	10.0	246	340	360
T	G23419	PER	Junin	9.2	210	310	323
J4	G21194	ARG	Jujuy	8.9	284	287	340
J1	G19895	ARG	Tucuman	8.0	254	337	362
M9	G12878	MEX	Guerrero	8.0	247	338	300
M18	G12855A	GTM	Jutiapa	7.1	273	292	337
M17	G23429	MEX	Puebla	7.0	266	344	303
M23	G12890	MEX	Michoacan	7.0	255	336	322
M16	G50506	GTM	Solola	6.4	240	313	356
M2	G23652	MEX	Puebla	6.2	248	353	311
A	G12857	PER	Junin	6.0	229	354	311
Ca	G12857	PER	Junin	6.0	244	415	253
H2	G12857	PER	Junin	6.0	256	357	254
M25	G12851	GTM	Santa Rosa	6.0	234	278	326
M1	G24390	MEX	Nayarit	5.0	241	359	339
M10	G10022	MEX	Durango	5.0	260	374	327
M7	G12881	MEX	Guerrero	5.0	270	361	314
M4	G23678	MEX	Jalisco	4.2	290	327	308
M15	G24365	MEX	Colima	4.1	249	353	328
M5	G12869A	MEX	Michoacan	4.0	293	360	304
J2	G23592	ARG		3.2	229	391	250
M3	G12873A	MEX	Morelos	3.0	269	348	327
mean ($n = 27$)				**7.7**	**251**	**334**	**322**
SD				**4.1**	**21**	**39**	**32**

[a] **Orig**: ARG, Argentina; BOL, Bolivia; CHI, Chile; COL, Colombia; GTM, Guatemala; MEX, Mexico, PER, Peru. **Province**. Cundina, Cundinamarca and Cocha, Cochabamba. [b] CP, crude protein (N × 5.7); NDF, neutral detergent fiber; and DM, average dry matter (925 ± 12 g/kg). [c] Weight of 100 seeds.

composition, (b) subunit patterns, (c) sequential hydrolysis by pepsin and pancreatin, (d) impact of heat treatment on phaseolin hydrolysis, and finally (e) theoretical availability of AA.

MATERIALS AND METHODS

Bean Cultivars. Cultivated *Phaseolus vulgaris* beans and wild beans utilized in this study were provided by the Bean Bank of the International Centre for Tropical Agriculture (CIAT, Cali, Colombia) (**Table 1**). These beans were used for phaseolin purification.

Isolation and Purification of Phaseolin. Phaseolin purification was conducted as previously described (*8, 14*). Briefly, whole seed beans were soaked in water (1:4, w:v) at 4 °C for 24 h, then frozen, and freeze-dried. The hull and embryo were removed with a scalpel. The cotyledon was then ground through a 0.5 mm mesh screen. The flour was extracted (1 g/20 mL) under acidic conditions (0.5 M NaCl and 0.025 M HCl, pH 2.0) for 1 h and then centrifuged at 20 000*g* for 20 min at room temperature. The supernatant fraction was mixed with five volumes of distilled water at 4 °C. This caused immediate precipitation of phaseolin. The suspension was centrifuged at 20 000*g* for 20 min at 4 °C. The precipitate was washed with distilled water, suspended in 0.5 M NaCl, and dialyzed against distilled water for 24 h at 4 °C. Then, it was centrifuged at 20 000*g* for 20 min at 4 °C, and the final precipitate was frozen and freeze-dried.

Phaseolin Treatment. All the phaseolins were studied either unheated or after heat treatment. Prior to heat treatment, half of the amount of purified phaseolin was dissolved in distilled water (100 mg/mL), and the pH was adjusted at 7.5. This solution was then autoclaved at 121 °C under a pressure of 15 psi for 15 min, frozen, and freeze-dried. These conditions for autoclaving were similar to those published in other studies (*8, 12, 15–17*) in order to allow comparisons between present and published data.

SDS-PAGE Analysis. The different phaseolin types were analyzed by using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) in order to separate their major constitutive subunits under reducing conditions and characterize them in terms of numbers and

Table 2. Characterization of Different Phaseolin Types

	cultivated beans								wild beans													
	I	M6	Pa	S	T	T01	X[a]	SD	H2	J1	J3	J4	K	L	M10	M2	M16	M17	M23	M25	X[a]	SD
									DH (%)[b]													
	71	75	91	58	71	96			58	96	73	67	57	93	83	73	65	73	82	89		
									N (g/kg DM)													
	155	153	138	154	155	149	**151**	**7**	144	145	149	151	138	141	138	145	137	148	147	138	**143**	**5**
									AA Composition (g/160 gN)													
ARG	51	54	59	50	53	59	**54**	**4**	57	57	51	56	59	57	60	53	57	58	56	58	**57**	**2**
HIS	32	36	39	33	35	39	**36**	**3**	38	39	35	38	40	37	40	37	40	36	36	38	**38**	**2**
ILE	41	44	48	44	44	48	**45**	**3**	47	48	44	46	48	45	47	44	45	44	43	45	**46**	**2**
LEU	71	76	81	78	74	81	**77**	**4**	79	81	74	79	81	77	81	75	79	77	81	77	**78**	**2**
LYS	61	66	70	68	65	70	**67**	**3**	68	69	63	68	71	67	72	66	71	66	70	67	**68**	**3**
MET	8	6	8	8	8	7	**8**	**1**	7	7	7	7	7	7	7	6	7	8	8	6	**7**	**1**
PHE	57	64	69	58	60	67	**63**	**5**	66	67	60	65	68	65	66	61	62	65	67	65	**65**	**2**
THR	31	33	36	26	34	34	**32**	**4**	34	34	26	33	35	34	36	33	35	34	34	34	**34**	**3**
VAL	45	48	49	47	48	49	**48**	**2**	50	51	47	49	51	48	52	47	51	49	49	48	**49**	**2**
ALA	38	43	44	34	37	42	**40**	**4**	44	45	40	43	46	44	46	42	45	43	45	45	**44**	**2**
ASP	101	108	119	116	105	116	**111**	**7**	111	114	107	112	115	110	113	105	109	106	115	109	**111**	**3**
CYS	2	2	2	3	3	2	**2**	**1**	2	2	3	2	3	2	4	3	4	2	2	2	**3**	**1**
GLU	122	146	157	147	146	157	**146**	**13**	151	148	137	148	155	144	156	144	149	149	148	144	**148**	**5**
GLY	32	34	38	36	35	36	**35**	**2**	37	36	34	36	39	37	38	35	38	35	35	37	**36**	**2**
PRO	34	41	45	39	36	44	**40**	**4**	43	43	36	41	44	44	48	43	46	43	43	45	**43**	**3**
SER	49	55	59	57	52	57	**55**	**4**	55	56	55	56	58	56	57	53	55	54	57	56	**56**	**1**
TYR	32	36	40	30	29	37	**34**	**4**	38	34	28	34	36	35	35	34	34	38	37	37	**35**	**3**
Total assayed	**806**	**891**	**963**	**874**	**864**	**946**	**891**	**57**	**935**	**936**	**850**	**919**	**959**	**910**	**959**	**884**	**934**	**910**	**930**	**915**	**920**	**31**

[a] Mean for phaseolin types in cultivated or wild beans. [b] DH, degree of hydrolysis at 360 min of hydrolysis for heated phaseolins (see also **Figure 2**).

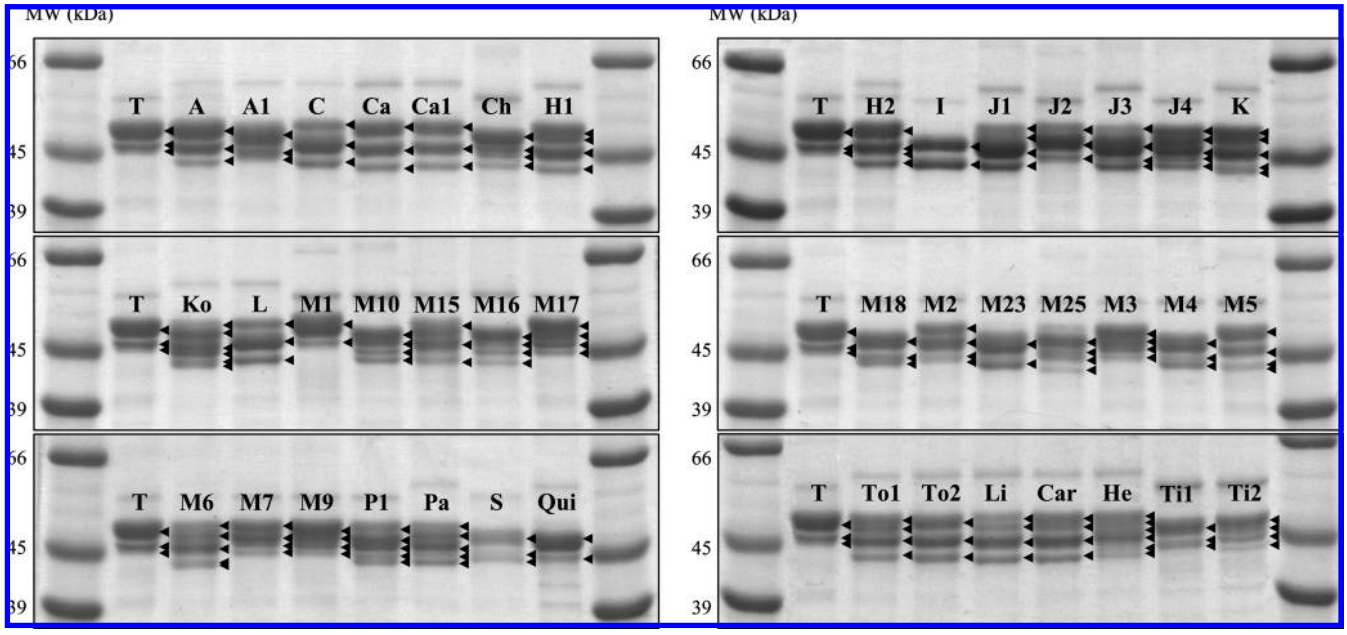

Figure 1. Electrophoretic subunit pattern of different phaseolin types in 1-D SDS-PAGE. Arrow heads indicate each subunit of a phaseolin type. MW markers are indicated on the left of the figure. See **Table 1** for the nomenclature of phaseolins.

relative MW (*16*). Briefly, the electrophoresis was carried out in stacking and separation gels with 5 and 10% of acrylamide, respectively. The samples were mixed (1:1, v:v) with 1 M Tris/HCl buffer, pH 6.8 (2.7 M glycerol, 139 mM SDS, and 0.15 mM blue bromophenol), and heated for 3 min at 100 °C. MW standards (14.2−66.0 kDa, MW-SDS-70L, Sigma) were also loaded in the first and last well of each gel. The phaseolin subunits were separated in 62.5 mM Tris/HCl buffer with 3.4 mM SDS at 40 mA for 3 h. After electrophoresis, the gels were stained with Coomassie brilliant blue (R-250, ref 27816, Sigma) in order to reveal phaseolin subunit banding patterns. The MW of each phaseolin subunit was determined by linear regression by using the protein MW standards run on each gel (*16*).

Enzymatic Hydrolysis. *Enzymes.* Porcine pepsin (EC 3.4.2.3.1, ref 107197, Merck) and pancreatin (EC 3.4.2.1.4, ref P1750, Sigma) enzyme preparations were used for studying the sequential hydrolysis of the phaseolin types in unheated or heated forms.

Hydrolysis Procedure. The in vitro hydrolysis protocol carried out under continuous stirring has been presented elsewhere (*17*). Briefly, each phaseolin was mixed with 0.1 N HCl, pH 2.0 (150 mg of protein in 15 mL of HCl), and preincubated for 30 min at 39 °C in a water bath. Then, pepsin (1 mL) was added to the medium with an enzyme-to-protein ratio of 1:67 (w:w). An aliquot (2 mL) was taken after 0, 30, and 120 min of pepsin hydrolysis. Then, 0.2 M phosphate buffered saline, pH 8.0, was mixed (1:1, v:v) with the remaining incubation media. Pancreatin (1 mL) was added at an enzyme-to-protein ratio of 1:30 (w:w). Aliquots were then taken 20, 120, and 240 min after pancreatin addition (i.e., at times 140, 240, and 360 min after pepsin addition). The incubation times for in vitro hydrolysis were those selected by other research groups (*18, 19*). All the phaseolins were hydrolyzed in triplicate. Casein was used as an internal control because of its availability and known susceptibility to proteolytic enzymes.

Characterization of Hydrolysis Products. One aliquot at each

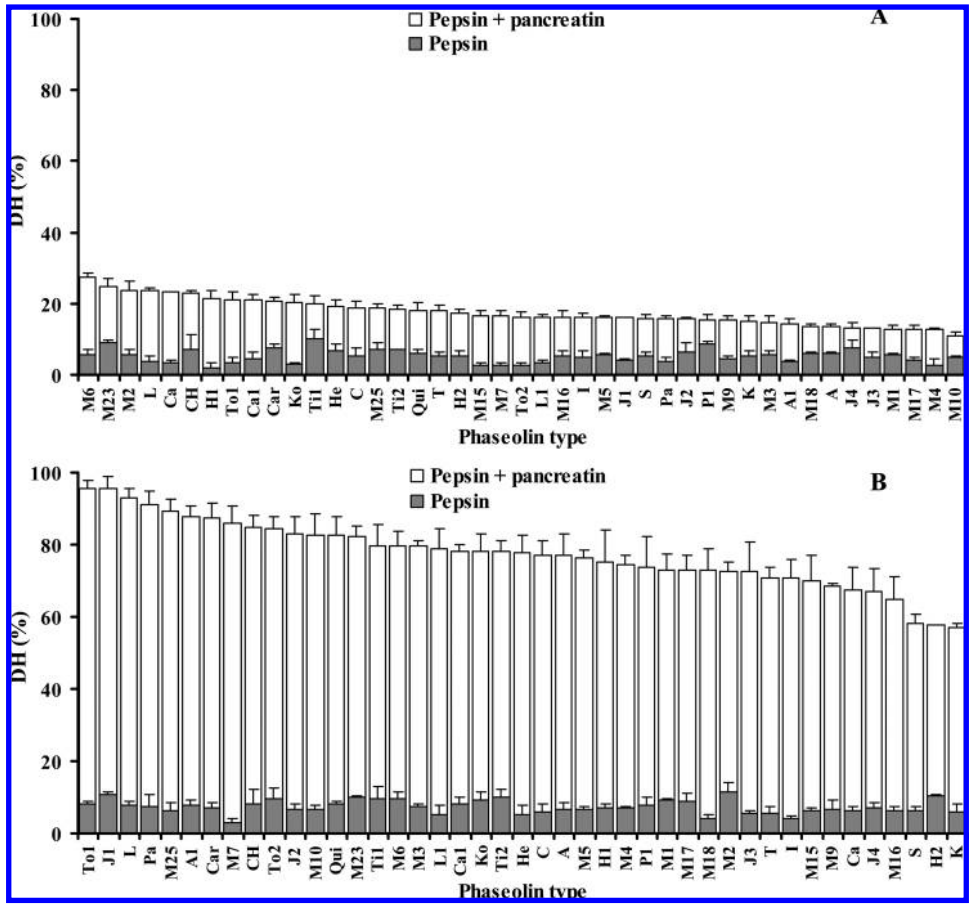

Figure 2. DH (%) of different phaseolins in unheated (A) and heated (B) form at 120 min (pepsin) and at 360 min (pepsin + pancreatin) of in vitro hydrolysis. Values are means and SEM for three measurements per phaseolin. Casein was used as a nonphaseolin control. Casein DH was 13.2 ± 4.9% at 120 min and 93.1 ± 2.4% at 360 min ($n = 8$). Phaseolin type effect: at 120 min, $P = 0.109$ and at 360 min, $P = 0.001$. Heat-treatment effect: at 120 min, $P = 0.001$ and at 360 min, $P = 0.001$. Phaseolin type by heat-treatment interaction: at 120 min, $P = 0.557$ and at 360 min, $P = 0.001$.

sampling time was immediately treated with trichloroacetic acid (TCA, 7.5%, w:v, final concentration). The supernatant was taken after centrifugation at 20 800*g* for 10 min to determine the soluble N by the Kjeldahl method.

Chemical Analysis. The beans were analyzed for dry matter (DM) (105 °C for 24 h), N (Kjeldahl method), neutral detergent fiber by using an Ankom fiber analyzer (Ankom Technology, Madecon, NY), and starch (amyloglucosidase), as presented elsewhere (*17*).

AA analysis was carried out on a subgroup of phaseolins (18 of 43 types). The selection of phaseolins to be analyzed for AA was made after their DH (see below for calculation) had been determined. We selected four unheated phaseolin having the highest DH (M6, M23, M2, and L) and four others having the lowest DH (M10, M17, J3, and J4). Similarly, we selected five heated phaseolins with a DH among the highest (To1, J1, L, P1, and M25) and five others with a DH among the lowest (K, H2, S, M16, and J4). We also analyzed AA in phaseolins T and I that have been studied in more details (*8, 12, 16, 17*). The AA were analyzed in duplicate by ion exchange chromatography by using a Biochrome 20 analyzer (Pharmacia Biotech Ltd., Cambridge, UK). Methionine and cysteine were determined by the same method after oxidation with performic acid before hydrolysis.

Calculations. *Comparisons of AA Profiles.* The comparisons between AA profiles assayed among phaseolins were determined by calculating the distance of χ^2 between profiles taken two by two (*20*). The χ^2 distance between two proteins is obtained by taking into account all the concentration differences between all the AA of the compared proteins. The composition of AA was expressed in percentage of the sum of the assayed AA.

$$\text{Distance of } \chi^2 = \Sigma(AA_{ij} - AA_{ik})^2/[(AA_{ij} + AA_{ik})/2]$$

where AA_{ij} and AA_{ik} are the AA i of the proteins j and k.

Degree of in Vitro Hydrolysis of Phaseolins. The DH of phaseolins was calculated as the ratio between TCA-soluble N in the aliquots over time and total N in the starting material, after correction for soluble N in the starting material, as follows:

$$DH_N = ([Ns_{(TX)}] - [Ns_{(T0)}]) \times 100/([N_{(Total)}] - [Ns_{(T0)}])$$

where $Ns_{(TX)}$ is N soluble in TCA at time X of the kinetics, $Ns_{(T0)}$ is N soluble at time 0, and $N_{(Total)}$ is total N in samples (*17, 18*).

Nutritional Quality of Phaseolins. The nutritional quality of the phaseolins could be estimated on the basis of either the first limiting AA or the essential AA. In agreement with that, we calculated the theoretical AA availability with the AA scores (AAS) and the theoretical protein digestibility corrected for AAS (PDCAAS) for the essential AA according to the following equations (*21*):

$$AAS = [AA_X \text{ phaseolin}_Y]/[AA_X \text{ reference pattern}]$$

$$PDCAAS \text{ phaseolin}_Y = AAS \text{ phaseolin}_Y \times DH\text{phaseolin}_Y$$

where the AA (g/160 gN) reference pattern corresponds to the requirements for children of 2−5 years old (*21*), and DH (%) is the DH of the phaseolin$_Y$ at 360 min of in vitro hydrolysis (120 min pepsin + 240 min pancreatin).

Statistical Analysis. An analysis of variance of the data was conducted in order to test the effect of the phaseolin type and phaseolin type by heat-treatment interaction at the final steps of pepsin (time 120 min) and pancreatin (time 360 min) hydrolysis, respectively. When the F-value of the analysis of variance was significant ($P < 0.05$), the means were compared by using the Duncan's multiple range test (*22*). To group phaseolins according to their DH, a cluster analysis was performed. All statistical analyses including cluster analysis were

Table 3. Number of Subunits in the Studied Phaseolin Types and Their Relative MW (kDa) As Determined by Using SDS-PAGE

	band number (in a decreasing order of MW)[a]					
phas[b]	1	2	3	4	5	6
			Cultivated Beans			
He	50.5–49.0 ± 0.55	48.6–47.8 ± 1.05	46.7–46.2 ± 1.30	45.6–44.8 ± 0.85	43.9–43.4 ± 0.05	42.0–41.5 ± 0.70
Ca1	52.2–48.5 ± 0.50	47.1–44.8 ± 0.20	43.5–42.8 ± 0.10			
Li	51.9–49.9 ± 0.35	49.2–47.7 ± 0.20	45.7–43.5 ± 0.75	42.4–41.5 ± 0.45		
To2	51.2–48.6 ± 0.45	46.9–45.0 ± 0.35	43.5–42.5 ± 0.35			
Ko	52.1–51.0 ± 0.40	50.5–48.6 ± 0.40	47.2–46.2 ± 0.75	45.7–44.9 ± 0.55	44.1–43.5 ± 0.25	43.2–42.9 ± 0.20
Pa	51.3–50.0 ± 0.65	49.4–48.5 ± 0.60	47.7–45.9 ± 0.50	44.6–44.3 ± 0.95	43.7–43.0 ± 0.70	
A1	51.4–47.9 ± 0.90	45.6–44.7 ± 1.15	44.5–43.8 ± 1.25			
Car	52.0–50.1 ± 0.85	49.6–47.6 ± 0.30	46.3–43.7 ± 0.70	41.7–41.1 ± 0.20		
To1	51.0–49.8 ± 0.25	48.9–47.8 ± 0.35	47.0–45.0 ± 0.35	43.7–42.7 ± 0.20		
Ti2	48.3–46.0 ± 0.60	44.9–44.2 ± 0.85	43.4–42.9 ± 0.70	42.3–41.8 ± 1.00		
H1	51.7–48.6 ± 0.50	48.3–47.2 ± 0.65	46.0–44.3 ± 0.60	42.6–41.7 ± 0.60		
Qui	49.3–44.8 ± 0.35	43.6–42.7 ± 0.40				
C	51.6–49.3 ± 0.85	48.1–45.0 ± 0.85	43.2–42.1 ± 0.20			
Ti1	48.7–46.2 ± 0.95	45.3–44.8 ± 1.05	44.1–43.7 ± 0.80			
S	50.1–47.7 ± 0.20	45.6–45.0 ± 0.45	44.8–44.1 ± 0.65			
I	48.9–46.7 ± 0.90	44.4–43.1 ± 0.55				
			Wild Beans			
Ch	51.0–47.5 ± 0.65	46.3–45.6 ± 0.15	45.1–44–7 ± 0.05	43.1–42.5 ± 0.15		
M6	51.3–49.8 ± 0.60	49.1–47.8 ± 0.15	46.6–44.5 ± 0.45	43.5–42.9 ± 0.20	42.5–42.0 ± 0.25	
K	50.9–48.7 ± 0.35	48.4–47.2 ± 0.20	45.3–44.1 ± 0.35	43.0–42.4 ± 0.30	42.1–41.7 ± 0.10	
L	52.7–50.2 ± 0.90	48.5–45.6 ± 0.50	44.3–43.1 ± 0.20			
J3	51.4–49.5 ± 0.20	48.9–45.5 ± 0.35	44.5–44.1 ± 0.60	43.5–42.9 ± 0.55		
P1	50.9–49.5 ± 0.45	48.6–47.0 ± 0.30	46.5–45.0 ± 0.75	43.7–43.1 ± 0.65	42.8–42.1 ± 0.65	
T	51.5–46.9 ± 0.40	45.5–44.7 ± 0.30	44.1–43.7 ± 0.20			
J4	51.7–49.7 ± 0.60	49.1–47.8 ± 0.80	46.8–45.4 ± 0.95	44.6–44.1 ± 0.80	43.6–42.6 ± 0.80	
J1	51.3–49.4 ± 0.75	49.0–47.8 ± 1.10	47.7–44.8 ± 1.05	43.2–41.7 ± 0.80		
M9	51.6–48.5 ± 0.40	47.9–46.2 ± 0.85	45.2–44.6 ± 1.15	44.4–43.9 ± 0.55		
M18	49.9–45.7 ± 0.50	44.0–43.4 ± 0.75	43.0–42.5 ± 0.65			
M17	53.6–49.8 ± 0.80	49.3–48.2 ± 0.25	47.4–46.7 ± 0.10	46.2–45.5 ± 0.30		
M23	49.0–44.8 ± 0.15	43.3–41.9 ± 0.35				
M16	50.9–48.6 ± 0.90	48.1–47.3 ± 0.70	45.7–45.0 ± 0.25	44.4–43.9 ± 0.65		
M2	51.4–48.5 ± 0.85	47.8–46.0 ± 1.55	45.4–44.9 ± 0.25	44.7–44.2 ± 0.20		
A	51.4–47.9 ± 0.60	46.8–46.1 ± 0.45	45.6–44.7 ± 0.90	43.6–42.8 ± 0.50		
Ca	51.8–47.8 ± 1.05	46.7–44.3 ± 0.55	42.9–41.9 ± 0.25			
H2	51.0–47.9 ± 0.30	47.0–45.9 ± 0.75	45.5–44.9 ± 0.50	43.5–42.7 ± 0.60		
M25	50.2–46.8 ± 0.55	46.0–45.2 ± 0.45	44.6–44.1 ± 0.15	43.1–42.5 ± 0.45		
M1	54.7–48.2 ± 0.65	47.0–46.1 ± 0.45				
M10	50.5–48.4 ± 0.65	47.7–46.3 ± 0.65	45.0–44.5 ± 0.65	43.7–43.2 ± 0.60		
M7	52.0–49.3 ± 0.20	48.5–46.8 ± 0.45	46.2–45.6 ± 0.95	44.2–43.6 ± 0.40		
M4	50.7–46.0 ± 0.80	45.1–44.2 ± 0.55	43.7–42.7 ± 0.50			
M15	52.2–49.5 ± 0.45	48.2–46.7 ± 1.05	45.3–43.6 ± 0.90			
M5	51.3–48.0 ± 0.75	47.0–45.9 ± 1.10	44.0–43.5 ± 0.75	43.1–42.6 ± 0.45		
J2	52.3–50.3 ± 0.30	49.2–46.6 ± 0.50	45.6–44.7 ± 0.60			
M3	51.5–48.7 ± 0.10	48.0–46.8 ± 0.25	46.0–45.4 ± 0.20	44.6–44.1 ± 0.15		

[a] Mean value ± standard deviation, $n = 3$. [b] Phas: phaseolin type (see **Table 1** for full identification).

performed by using the General Linear Model procedure of Statistical Analysis Systems, statistical software package version 8.0 (SAS Institute Inc., Cary, NC).

RESULTS

Biochemistry of Phaseolin Types. *Bean Characteristics and Composition.* The seeds of cultivated beans were heavier than those of wild beans (45 ± 21 and 8 ± 4 g/100 seeds, respectively). A moderate variability in the nutritional composition among bean materials used for phaseolin purification was observed (**Table 1**). The content in protein (241 ± 25 g/kg DM) and starch (356 ± 60 g/kg DM) of bean presented coefficients of variation of 11 and 17%, respectively. However, wild beans had higher protein (251 ± 21 g/kg DM) and neutral detergent fiber (NDF, 334 ± 39 g/kg DM) contents compared to cultivated beans (protein, 224 ± 20 g/kg DM and NDF, 232 ± 33 g/kg DM). Protein was negatively correlated with starch ($r = -0.49$, $P = 0.001$) and methionine ($r = -0.66$, $P = 0.002$) content. Starch content was higher in cultivated beans (391 ± 63 g/kg DM) than in wild beans (322 ± 32 g/kg DM, $P = 0.005$).

AA Composition and Profiles. **Table 2** shows the AA composition of different phaseolin types. Variation in AA composition among the analyzed phaseolins ($n = 18$) was quite low (distance of $\chi^2 = 4.8 \pm 2.7$). As a comparison, the χ^2 distance between the average AA composition of the analyzed phaseolins ($n = 18$) and bovine caseins was determined to be 288. Nevertheless, some phaseolins had AA profiles somewhat different from the average AA composition profile of the others (S phaseolin, $\chi^2 = 14$ and J3 phaseolin, $\chi^2 = 8$). The differences were mainly due to the variability in the content of cysteine (CYS, 2.9 ± 0.7 g/160 g N, coefficient of variation (CV) = 23%) and methionine (MET, 7.9 ± 1.0 g/160 g N, CV = 11%). The content of the other AA was essentially similar between phaseolins (CV = 1.7−6.7%).

Phaseolin Subunits. The subunit pattern of the purified phaseolins in SDS-PAGE is shown in **Figure 1**. The number of subunits among phaseolins varied from 2 to 6 bands with specific MW for each subunit ranging from 54.7 to 41.1 kDa (**Table 3** and **Figure 1**). Phaseolins with 2, 3, 4, 5, and 6

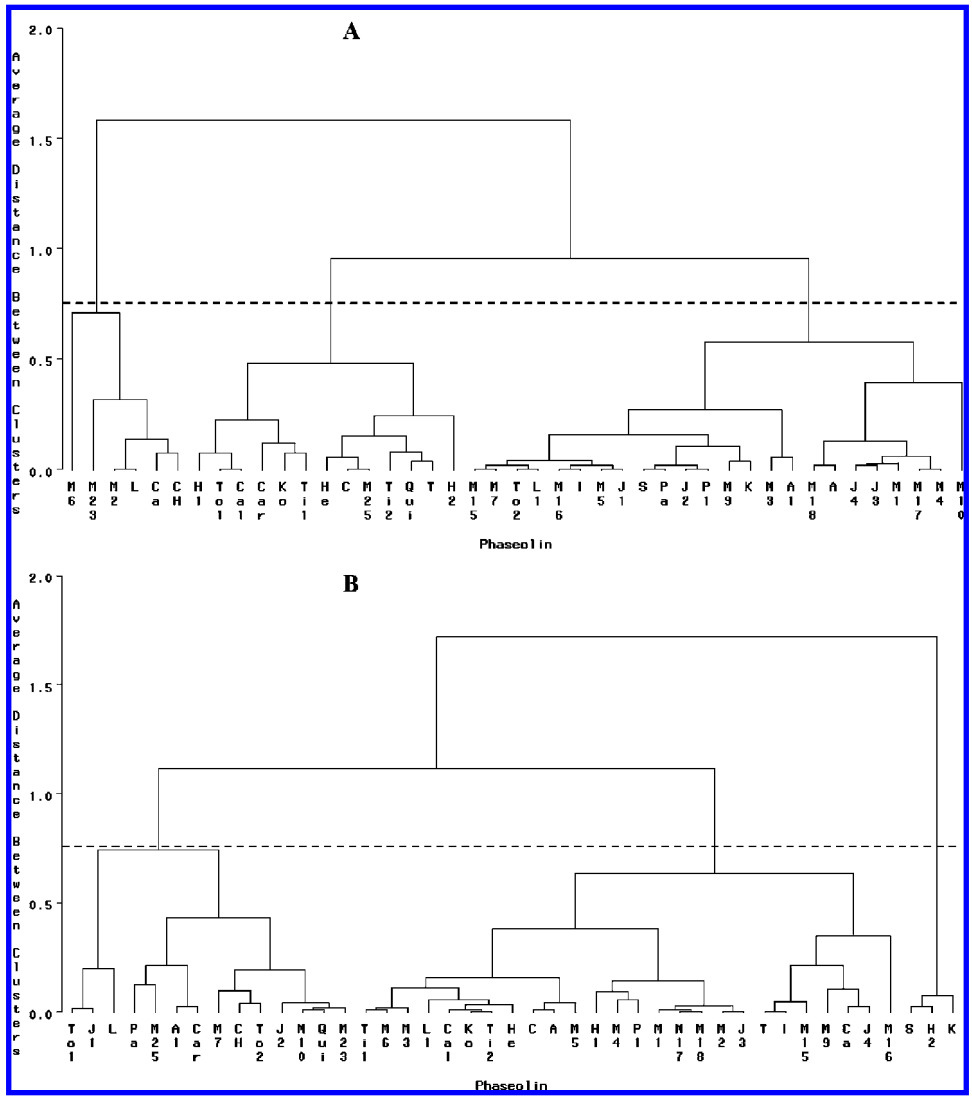

Figure 3. Cluster analysis of phaseolin types at 360 min of enzymatic in vitro hydrolysis (120 min pepsin + 240 min pancreatin) for unheated (A) and heated (B) phaseolins.

subunits represented 9.3, 30.2, 44.2, 11.6, and 4.7% of all the phaseolins studied here, respectively. Those with 3 and 4 subunits accounted for nearly 75% of the total.

In Vitro Hydrolysis. No effect ($P > 0.05$) of the phaseolin type and phaseolin type by heat-treatment interaction was observed for DH at 120 min of pepsin hydrolysis for unheated (5.2 ± 1.8%) and heated (7.5 ± 2.0%) phaseolins. A high correlation ($DH_{360} = 0.70\ DH_{240} + 30.29$; $n = 43$; $r = 0.82$) was found between DH values obtained at 240 and 360 min for heated phaseolins. Therefore, only the results for 360 min, which represent phaseolin hydrolysis potential, are reported here. However, at 360 min of hydrolysis, the phaseolin type by heat-treatment interaction was significant ($P < 0.001$), and a high variability in enzymatic susceptibility among unheated (DH from 11.0 ± 2.0 to 27.4 ± 1.8%, $P < 0.001$) and heated (DH from 56.9 ± 2.5 to 95.7 ± 3.5%, $P < 0.001$) phaseolins was observed (**Figure 2A** and **B**, respectively). Heat treatment increased the DH of phaseolins by 2.7- to 7.5-fold ($P < 0.001$).

To group the broad number of phaseolin types and the DH variability, a cluster analysis was conducted (**Figure 3A** and **B**). It confirmed the high variability of DH among unheated and heated phaseolins. Heat treatment completely changed phaseolin clustering patterns. An average distance of 0.75 between clusters in the analysis was selected in order to limit the number of groups to three (high, medium, and low DH) for a better representation of DH variability among phaseolin groups (**Figure 4**). These groups represented 14, 30, and 56% of phaseolins for unheated phaseolins, respectively, and 33, 60, and 7% for heated phaseolins, respectively. The differences among these cluster groups for unheated and heated phaseolins were first observed after pancreatin addition, and they persisted until the end of the hydrolysis kinetics (**Figure 4**).

Estimated Nutritional Quality. The theoretical nutritional quality of phaseolins was determined by AAS and PDCAAS. Sulfur AA was found to be the limiting AA in phaseolin, because its calculated AAS led to requirement coverage of 39 ± 3% only (**Table 4**). Some phaseolins presented higher values than the average (S and M16 with 44 and 43% of requirements, respectively). The calculation of PDCAAS confirmed that sulfur AA (29 ± 5%) is the main limiting AA and showed that threonine would be the second limiting AA (THR, 74 ± 15%; **Table 4**). The PDCAAS for sulfur AA showed that Pa, To1, J1, M10, and M23 phaseolins had the highest values (38, 35, 36, 34, and 34%, respectively). The first three phaseolins were influenced by DH only, whereas the last two phaseolins were influenced by both the content of sulfur AA and DH. The phaseolins with the highest DH (To1, J1, L, and Pa with 96, 95, 92, and 95%, respectively) could almost cover the require-

ments for threonine. The theoretical AA availability calculated with PDCAAS was covered for histidine, isoleucine, phenylananine + tyrosine, and valine in almost all phaseolins. Nevertheless, the theoretical availability for these AA was not covered for S, H2, and K phaseolins. Leucine and lysine requirements were only covered for phaseolins with the highest DH (To1, J1, L, Pa, and M25) or for phaseolins with both a high content of these AA and a high DH (M10 and M23).

DISCUSSION

Phaseolin Diversity. Phaseolin is coded by a gene cluster which may have appeared by successive duplication and divergence (e.g., insertions−duplications and nucleotide substitutions) of an ancestral gene (*10*). Moreover, the co- and post-translational modifications at the DNA sequence level (precursor α and *β*) generated different cleavages of a signal peptide and glycosylations. This led to the existence of a similar group with slightly heterogeneous phaseolin polypeptides (*10, 11*). This information may explain the difference in subunit patterns between phaseolins found here, with phaseolin subunits MW ranging from 54.7 to 41.1 kDa. Each phaseolin molecule was found with a number of subunits varying between 2 and 6. Phaseolin profiles varying from 3 to 6 subunits and MW ranging from 54.4 to 45.6 kDa in SDS capillary gel electrophoresis were observed for 11 phaseolins (*9*). Eight subunits with different MW were distinguished among these phaseolins.

Mass spectrometry identification of phaseolin subunits showed differences in the patterns of precursors among S, T, and I phaseolin types (α and *β* for S; α, *β*, and *β* for T; and *β* and *β* for I) (*16*). The S, T, and I phaseolin subunits showed differences in MW of subunits for a same precursor type. Fukuda et al. (*23*), by screening wild soybean lines, found variations in AA sequences of the subunits that affected the electrophoretic migration of *β*-conglycinin and glycinin. It would be of interest to determine the α or *β* precursor origin of the phaseolin subunits identified here in order to search for relationships between phaseolin subunit molecular origin and susceptibility to proteolysis.

Susceptibility of Phaseolins to Proteolysis. The time taken for in vitro hydrolysis (pepsin 120 min + pancreatin 240 min) was chosen because it is the most common value used for in vitro kinetic hydrolysis (*18, 19*). Moreover, the stomach half-emptying time is 123 ± 47 min (*24*), and the oro−cecal transit time is around 300 min (*25*) in healthy humans. The data presented here for DH are probably close to the overall hydrolysis potential of the studied phaseolins.

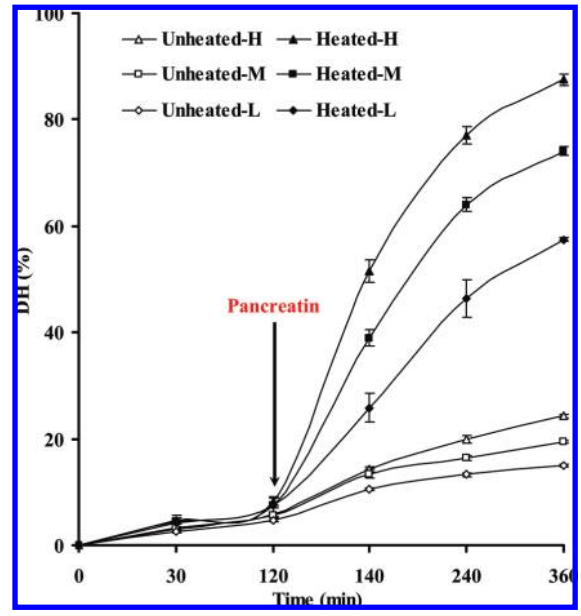

Figure 4. Changes in DH (%) over time after sequential in vitro hydrolysis with pepsin (0–120 min) and pancreatin (120–360 min) for groups of phaseolins as defined after cluster analysis for DH. DH: high (H), medium (M), and low (L).

Until this work, no evidence was available on the variability of DH for a large collection of phaseolins. Our results confirm the high resistance of unheated phaseolin to proteolytic attack as reported previously (*6, 7, 12*). Resistance to proteolysis was high regardless of the phaseolin subunit composition. However, it varied widely among phaseolins for both unheated (DH from 11 to 27.4%) and heated (DH from 56.9 to 95.7%) forms. This high variability may be ascribed to differences in protein composition and, therefore, in tertiary and quaternary structures.

Table 4. AAS and PDCAAS of Different Phaseolin Types

	cultivated beans								wild beans													
	I	M6	Pa	S	T	To1	X[a]	SD	H2	J1	J3	J4	K	L	M2	M10	M16	M17	M23	M25	X[a]	SD
												DH (%)[b]										
	71	75	91	58	71	96			58	96	73	67	57	93	83	73	65	73	82	89		
												AAS (%)[c]										
HIS	169	189	206	171	184	205	**187**	**16**	200	205	183	199	201	197	197	212	210	189	192	199	**199**	**8**
ILE	146	156	170	157	157	173	**160**	**10**	170	173	156	165	170	162	156	168	162	159	155	161	**163**	**6**
LEU	107	116	123	119	112	123	**117**	**6**	120	123	112	120	123	116	113	122	120	117	123	117	**119**	**4**
LYS	105	114	121	117	112	120	**115**	**6**	118	119	109	117	122	115	113	125	122	113	120	116	**117**	**5**
MET+CYS	42	32	42	44	42	36	**40**	**5**	37	37	39	37	40	35	38	42	43	40	42	32	**39**	**3**
PHE +TYR	140	159	172	140	142	165	**153**	**14**	164	160	139	158	165	159	152	161	152	164	165	162	**158**	**8**
THR	92	96	105	77	101	100	**95**	**10**	101	99	75	96	103	99	98	106	104	101	101	100	**99**	**8**
VAL	128	136	140	134	137	141	**136**	**5**	144	146	136	140	147	138	135	149	146	139	139	137	**141**	**5**
												PDCAAS (%)										
HIS	119	142	187	99	130	196	**146**	**38**	115	196	133	134	119	183	143	175	136	138	158	178	**151**	**27**
ILE	103	117	155	91	111	165	**124**	**30**	98	165	114	111	97	151	113	139	105	116	128	144	**123**	**22**
LEU	76	87	112	69	79	118	**90**	**20**	69	117	81	81	70	108	82	101	78	85	101	105	**90**	**16**
LYS	75	86	110	68	79	115	**89**	**19**	68	114	79	78	69	107	82	103	79	83	99	103	**89**	**16**
MET+CYS	29	24	38	26	30	35	**30**	**5**	21	36	28	25	23	32	28	34	28	29	34	29	**29**	**5**
PHE +TYR	99	119	156	81	101	158	**119**	**32**	95	151	101	106	94	148	111	133	99	119	135	145	**120**	**22**
THR	65	72	95	45	71	96	**74**	**19**	58	95	55	65	59	92	71	87	67	74	83	89	**75**	**14**
VAL	91	102	128	78	97	135	**105**	**22**	83	139	99	94	84	128	98	123	95	101	115	123	**107**	**18**

[a] Mean for phaseolin types in cultivated or wild beans. [b] DH, degree of hydrolysis at 360 min of hydrolysis for heated phaseolins (see also **Figure 2**). [c] Contribution of the phaseolin for one of the essential AA that covers the requirements of children of 2−5 years old (*21*).

Slight differences in tertiary structures of the monomer cause distinct quaternary structures (*26*). However, the present work was not designed for investigating the relationships between phaseolin structure and susceptibility to proteolysis. The glycosylations (not studied here) could also affect the proteolytic susceptibility of the phaseolins (*27*). Previously, we showed differences in DH among three phaseolin types (S, 58%; T, 71%; and I, 71%) (*12*). Deshpande and Nielsen (*7*) worked on phaseolins from 17 common bean varieties, some of them differing in their subunit composition. They found that all tested varieties generated similar major breakdown products after enzyme attacks. However, this work with SDS-PAGE provided only qualitative information on the variability in phaseolin susceptibility to proteolysis in vitro.

Improvement of Phaseolin Hydrolysis by Heat Treatment. The susceptibility to enzymatic hydrolysis of phaseolins increased by 2.7- to 7.5-fold following heat treatment. This improvement appeared to be independent of proteolysis susceptibility in the unheated state. Heat treatment influences structural changes and favors enzymatic hydrolysis by decreasing the percentage of α-helixes while increasing random structures in the molecule (*28*). Most phaseolin bands had drastically reduced intensities in SDS-PAGE after in vitro hydrolysis (*7, 28, 29*). Heat treatment increased the variability in DH among unheated phaseolin types, as reported previously for S, T, and I phaseolins (*12*). Differences in thermal stability, surface hydrophobicity, solubility, heat-induced association of individual subunits (α, α′, and β) and their combination (α3, α2β1, α′2β1, etc.) in soybean β-conglycinin variants were observed (*30, 31*). Therefore, it might be possible that the differences previously reported in the profile of subunits precursors (α and β) in phaseolins (e.g., αβ for S, α$\beta\beta$ for T, and $\beta\beta$ for I (*16*)) could affect differentially their thermal stability or surface hydrophobicity with the subsequent effects on their susceptibility to proteolysis as found here.

Improvement of Bean Nutritional Quality. Little differences among phaseolins in theoretical nutritional quality as estimated with AAS indicator were found for the indispensable AA (average CV = 5%) in the present work. However, the differences were higher for PDCAAS indicator (average CV = 19%), showing that the nutritional quality of heated phaseolins was influenced more by DH than by AA composition. In a previous study, we did not find any differences for AAS and PDCAAS indicators among purified S, T, and I phaseolins (*17*), probably because all three phaseolins displayed a low DH after heat treatment. A bean cultivar genetically modified to express different phaseolin types (S, T, or I) was studied to investigate whether the phaseolin type could influence proteolysis susceptibility and nutritional value of total bean protein. Similar differences in in vitro hydrolysis among bean lines and among purified phaseolins were observed, suggesting that the differences in DH among bean lines were correlated with the susceptibility of the phaseolin type to proteolysis (*12, 17*).

Cluster analysis of heated phaseolins conducted here showed that S, T, and I phaseolins studied previously (*12, 17*) were among the 10 phaseolins with the lowest DH of the collection. The present results suggest that it would be possible to improve the nutritional value of beans by selecting phaseolins with a high DH after heat treatment (e.g., To1, J1, Pa, and L) for a breeding program.

In conclusion, differences in enzymatic susceptibility among unheated and heated phaseolins were observed. Heat treatment increased the DH of phaseolins on average by 60 points but with large differences among phaseolins. Small differences in AA composition profiles and in the corresponding estimated nutritional quality of phaseolins were noted; the present data strongly suggest that selecting common beans on the basis of the susceptibility of their phaseolin to in vitro proteolysis after heat treatment would be the most effective way to improve the nutritional value of beans for humans. Accessing the present phaseolin collection provides new opportunities for investigating relationships between phaseolin structure and susceptibility to proteolysis more deeply.

ABBREVIATIONS USED

AA, amino acid; AAS, AA scores; DH, degree of hydrolysis; MW, molecular weight; PDCAAS, protein digestibility corrected for AAS.

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