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Transport of Amino Acids from in Vitro Digested Legume Proteins or Casein in Caco-2 Cell Cultures

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Purified legume storage proteins (chickpea 11S and 7S globulins, faba bean globulins, and lupin globulins) and casein (casein) were subjected to an in vitro enzyme (pepsin + pancreatin) digestion process. Protein digests were then used in a bicameral Caco-2 cell culture system to determine amino acid transport across the cell monolayer. With digests from legume proteins, absolute amounts of aspartate, glycine, and arginine transported were higher than those found in digested casein, whereas amounts of glutamate, proline, tyrosine, valine, and lysine were lower. However, proportions of amino acids in the basolateral chamber as compared with amounts added in the apical chamber were lower than casein controls for all amino acids except cystine. Results confirm previous in vivo observations that amino acids from legume proteins are probably absorbed at rates different from those in other proteins of animal origin such as casein.

KEYWORDS: Legume proteins; Caco-2 cells; amino acid transport

INTRODUCTION

In a recent review on the nutritional value of proteins from different food sources including legumes, cereals, and others, Friedman concluded that the development of improved protein sources to feed the growing world population will be facilitated by parallel developments in our understanding of factors governing the digestion, absorption, and utilization of amino acids from different protein sources (*1*). A number of works have been published in recent decades on the nutritional utilization of legume seeds, as these foodstuffs contain 20−50% protein and therefore constitute a promising source of vegetable protein. Lower protein digestibility and the presence of antinutritional factors (ANF) have been traditionally claimed in the literature as the main reasons for their lower nutritional value compared with other protein sources. However, recent data suggest that even though in vivo digestibility of extracted legume proteins is comparable or even higher than that of control proteins (lactalbumin and casein), diets based on purified legume proteins do not support the growth of rats at the same rates as control proteins and induce changes in N metabolism, organ relative weights, and plasma amino acid concentrations. The reasons for these effects remain unclear, but one possible cause might be that, although their intestinal enzyme digestibility is not different from controls, legume proteins might be absorbed at rates different from those of other proteins of animal origin such as casein or lactalbumin (*2*). Therefore, even though the nutritive value of any protein ultimately depends on its content of essential amino acids, differences in absorption and/or utilization of amino acids would affect that theoretical nutritional value.

Caco-2 cell cultures are currently being utilized as in vitro models for absorption studies involving minerals (*3*), nucleic acids (*4*), proteins (*5*), and protein hydrolysates (*6*) because monolayers of Caco-2 cells possess many of the properties of mature intestinal absoptive epithelial cells (*7*). On the other hand, previous research with rats (*2*) suggests that legume proteins are absorbed and utilized in ways different from those of animal origin usually utilized as controls (lactalbumin and casein). Therefore, the aim of the present work was to investigate how this in vitro system compares with the in vivo (rat) model with respect to amino acid absorption.

MATERIALS AND METHODS

Protein Purification. Chickpea (*Cicer arietinum* var. kabuli), faba bean (*Vicia faba* var. minor), and lupin (*Lupinus angustifolius* sweet var.) seeds were purchased locally. Casein and reagents used were from Sigma Chemical Co. (St. Louis, MO). Globulins were purified from chickpea, faba bean, and lupin seed meals after extraction at pH 8.0 in 0.2 mol/L borate buffer overnight at 4 °C. The extract was centrifuged (48000*g*, 4 °C, 30 min, Sorvall), the supernatant was treated with $(HN_4)_2SO_4$ (760 g/L) and centrifuged again in the same conditions, and the precipitate was resuspended and extensively dialyzed against distilled water. The fraction insoluble in water after dialysis was freeze-dried and taken as the storage globulins fraction. Chickpea 11S and 7S globulins were obtained as described in Danielsson (*8*). Briefly, the pH of the extracted globulins fraction after extraction in pH 8.0 buffer was lowered to 4.5 with glacial acetic acid under continuous stirring, and both supernatant (7S globulins) and sediment (11S globulins) were recovered after centrifugation, dialyzed extensively against distilled water, and freeze-dried.

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In Vitro Digestion of Proteins. The procedure followed for protein digestion in vitro was as described by Glahn (*9*) with some modifications. Enzymes were obtained from Sigma Chemical Co. Proteins (200 mg) were suspended in 8 mL of 120 mmol/L NaCl in 50 mL screw-cap sterile plastic tubes, mixed with a tube shaker, and allowed to stand at room temperature for 15 min. The pH was then adjusted to 2.0 with 5.0 mol/L HCl, the volume brought to 10 mL with NaCl solution, and 0.1 mL of pepsin (P-7000) solution (0.1 g/2.5 mL of 0.1 mol/L HCl) added to each sample. The tubes were placed in a shaking (55 oscillations/min) water bath at 37 °C for 60 min. For the intestinal digestion step, the pH was raised to 6.0 with 1 mol/L $NaHCO_3$ dropwise, and 2.5 mL of pancreatin−bile extract (P-3292 and B-8756, respectively) mixture (0.1 g of pancreatin + 0.6 g of bile extract in 50 mL of 0.1 mol/L $NaHCO_3$) was added. The pH was adjusted to 7.5 with NaOH and the volume brought to 15 mL with 120 mmol/L NaCl; tubes were capped and placed in a shaking water bath at 37 °C for 120 min. After hydrolysis, enzymes were inactivated by incubating the digest at 100 °C for 4 min in a water bath. The tubes were then allowed to cool and centrifuged (3913*g*, 4 °C, 30 min, Hettich, Universal 30RF). Aliquots (1.5 mL) of the supernatants after centrifugation were stored at −80 °C until used for (a) amino acid determination [proportions (percent) of amino acids in this fraction with respect to amounts added for protein digestion were determined after hydrolysis] or (b) cell culture experiments.

Cell Culture. Caco-2 cells were purchased from the European Collection of cell cultures (ECACC) at passage 40 and used in experiments at passages 42−50. All cell culture media and cell culture-grade chemicals were obtained from Sigma Chemical Co. Culture flasks were purchased from Corning Costar (Cambridge, MA). Cells were grown in 75-cm^2 plastic flasks containing high-glucose Dulbecco's modified minimal essential medium (DMEM), with heat-inactivated fetal bovine serum (15%), $NaHCO_3$ (3.7 g/L), nonessential amino acids (1%), HEPES (15 mmol/L), bovine insulin (0.1 UI/mL), and 1% antibiotic−antimycotic (Sigma, A9909) solution. The cells were maintained at 37 °C in an incubator in an atmosphere of air/CO_2 (95:5) at 90% humidity, and the medium was changed every 2 days. Trypsinization and seeding of cells into bicameral chambers (Transwell, 24 mm diameter, 4.7 cm^2 area, 3 μm pore size, Costar) were performed as described elsewhere (*10*, *11*).

Amino acid transport experiments were carried out 21 days after initial seeding. Spent culture medium was aspirated from the apical and basolateral chambers, and apical and basolateral cell surfaces of the monolayer were washed three times with Ca^{2+}- and Mg^{2+}-free HBSS at 37 °C. Then, 2.5 mL of transport solution (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 7) was added to the basolateral chamber, and aliquots of protein digests (1.5 mL, four replicates per protein tested, see above) were added to the apical chamber. DMEM (1.5 mL) without fetal bovine serum was used as negative control. Cell cultures were incubated at 37 °C in humidified air/CO_2 atmosphere for 4 h. As an index of cell viability and monolayer integrity, transepithelial electrical resistance (TEER) was recorded from 48 h before the start of the experiment (238 ± 7 Ω/cm^2) until the proteins were added (251 ± 5 Ω/cm^2). At the start of the experimet, immediately after proteins were loaded (time 0), TEER was 165 ± 10 Ω/cm^2, and after 4 h it was 172 ± 14 Ω/cm^2, which was not different from time 0. The buffer in the basolateral chambers was then removed, placed in sterile plastic tubes, and freeze-dried.

Transport of amino acids across the monolayer was calculated as $(B/A) \times 100$, where B stands for absolute amounts (micrograms) of each amino acid determined in basolateral chambers after 4 h of incubation and A for initial absolute amounts at time 0 in apical chambers for the different protein digests tested.

Analytical Determinations. Amino acids in proteins, protein digests, and freeze-dried buffer in basolateral chambers were determined after hydrolysis in 6 mol/L boiling HCI (2 mL/5 mg of protein) for 18 h; sulfur-containing amino acids were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively (*12*). Amino acids were determined by HPLC (Waters) using the Pico Tag method after derivatization with phenyl isothiocyanate.

Table 1. Amino Acid Composition (Milligrams per Gram) of Control (Casein) or Purified Legume Proteins

	protein[a]				
	CAS	CP11S	CP7S	VFG	LMG
Asp	33	84	122	105	89
Glu	157	129	174	164	169
Ser	42	43	60	36	30
Gly	15	29	33	36	28
His	15	21	27	22	20
Arg	22	73	76	83	95
Thr	34	27	34	28	26
Ala	22	33	35	34	19
Pro	60	40	37	44	33
Tyr	51	22	23	26	30
Val	51	36	45	45	29
Met + Cys	27	10	16	15	17
Ile	38	36	48	44	38
Leu	61	64	101	69	65
Phe	32	51	63	38	33
Lys	49	51	89	48	37

[a] CAS, casein; CP11S, chickpea 11S globulin proteins; CP7S, chickpea 7S globulin proteins; VFG, faba bean globulins; LMG, lupin meal globulins.

Statistical Analysis. The results were subjected to one-way analysis of variance and Tukey's multiple comparison test for differences between means ($P < 0.01$) (Minitab Statistical Software Package, Minitab, New York, NY).

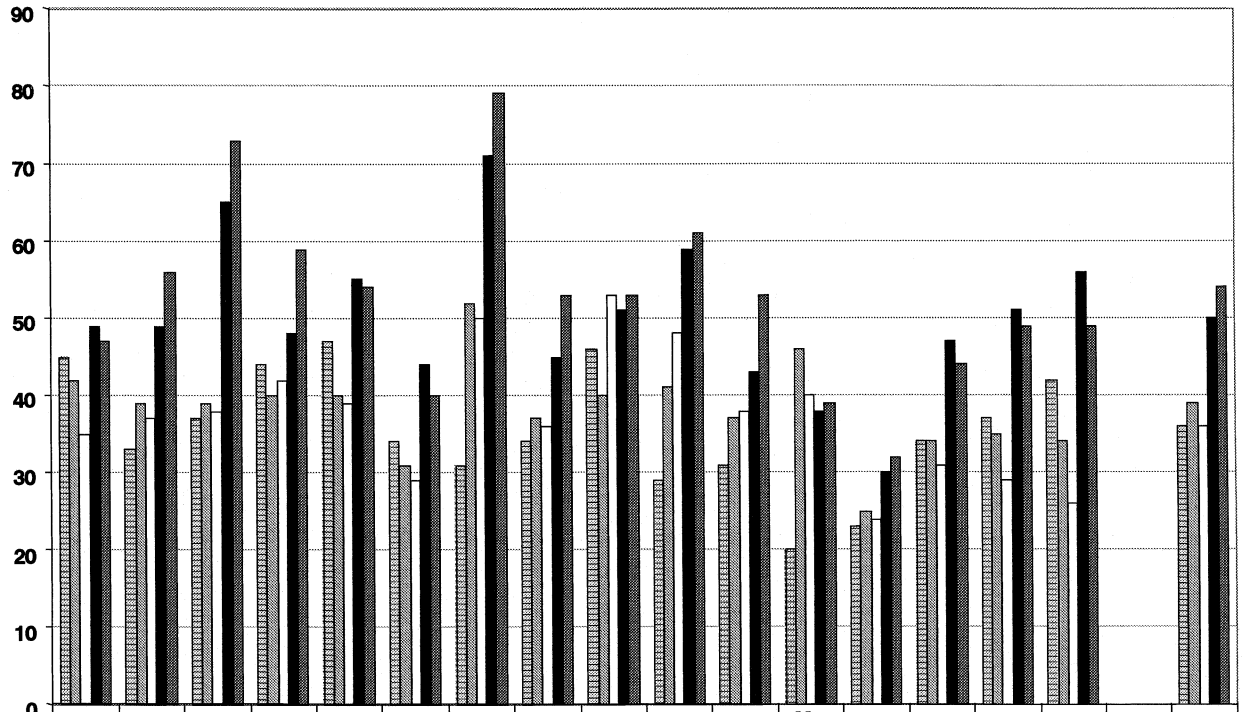
RESULTS

Enzyme Hydrolysis of Proteins. The amino acid composition of the proteins utilized in the current study is reported in **Table 1**. For most amino acids (except Lys in CP11S and CP7S), amounts recovered in the soluble fraction after in vitro enzyme (pepsin + pancreatin) hydrolysis with respect to initial amounts (**Figure 1**) were not different from or higher ($P < 0.01$) than casein for all legume proteins tested (chickpea 11S and 7S, faba bean, and lupin globulins).

Amino Acid Transport in Cell Cultures. Amounts of amino acids incorporated in the apical chambers of the different wells for the proteins tested are shown in **Table 2**. Those values depended mainly on the amounts digested for each protein after the in vitro enzyme digestion process. Values for most amino acids were not different or higher for legume proteins compared to casein, which also resulted in higher total values. Amino acid transport was expressed as absolute amounts (micrograms) of amino acids transported to the basolateral chamber per well (**Table 3**) and as proportions (percent) of amino acids transported per well from the initial amount added to the apical chamber (**Figure 2**). Except for aspartate, glycine, arginine, and alanine, amounts of amino acids recovered in the basolateral chambers (**Table 3**) of legume proteins after 4 h of incubation were not different from or lower ($P < 0.01$) than casein. On the contrary, transport values of individual amino acids (**Figure 2**), except cystine, were lower ($P < 0.01$) than controls in legume proteins. The total amount of amino acids in DMEM basolateral chambers was 526 ± 7 μg.

DISCUSSION

The first step for a feed or food protein to be utilized by the host is its degradation and absorption in the gastrointestinal tract. It is generally assumed that the presence of ANF and the slower digestion rate of legume as compared to animal proteins could explain their lower nutritional efficiency. Nevertheless, this conclusion is most of the time based on experiments in which the whole seed meal was used in the diet. When purified proteins



	Asp	Glu	Ser	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met + Cys	Ile	Leu	Phe	Lys		Average
CAS [1]	45^{a}	33^{a}	37^{a}	44^{a}	47	34	31^{a}	34^{a}	46	29^{a}	31^{a}	20^{a}	23	34^{a}	37^{a}	42^{b}		36
CP11S	42^{a}	39^{a}	39^{a}	40^{a}	40	31	52^{b}	37^{a}	40	41^{ab}	37^{a}	46^{b}	25	34^{a}	35^{a}	34^{a}		39
CP7S	35^{b}	37^{a}	38^{a}	42^{a}	39	29	50^{b}	36^{a}	53	48^{ab}	38^{a}	40^{b}	24	31^{a}	29^{a}	26^{a}		36
VFG	49^{a}	49^{b}	65^{b}	48^{ab}	55	44	71^{c}	45^{ab}	51	59^{b}	43^{ab}	38^{b}	30	47^{b}	51^{b}	56^{b}		50
LMG	47^{a}	56^{b}	73^{b}	59^{b}	54	40	79^{c}	53^{b}	53	61^{b}	53^{b}	39^{b}	32	44^{ab}	49^{b}	49^{b}		54

Figure 1. Amounts of amino acids recovered in the soluble fraction after in vitro enzyme (pepsin + pancreatin) hydrolysis with respect to initial amounts. CAS, casein; CP11S, chickpea 11S globulin proteins; CP7S, chickpea 7S globulin proteins; VFG, faba bean globulins; LMG, lupin meal globulins. [a–c]Means in the same column with different superscript letters differ significantly ($P < 0.01$).

Table 2. Amounts of Amino Acids Added to the Apical Chambers (Micrograms per Well) of Caco-2 Cell Cultures for the Different Proteins

	protein[a]				
	CAS	CP11S	CP7S	VFG	LMG
Asp	303	700	846	1034	852
Glu	1037	1003	1287	1614	1908
Ser	312	335	458	468	334
Gly	128	239	280	343	334
His	141	169	209	243	219
Arg	146	449	437	726	770
Thr	210	286	338	400	414
Ala	150	242	250	309	205
Pro	550	323	395	451	520
Tyr	290	185	219	308	364
Val	317	260	343	388	310
Met	72	55	88	79	91
Cys	34	35	39	36	42
Ile	174	182	231	269	241
Leu	421	433	626	649	576
Phe	241	352	372	385	325
Lys	412	352	466	541	362
total	4940	5602	6887	8245	7974

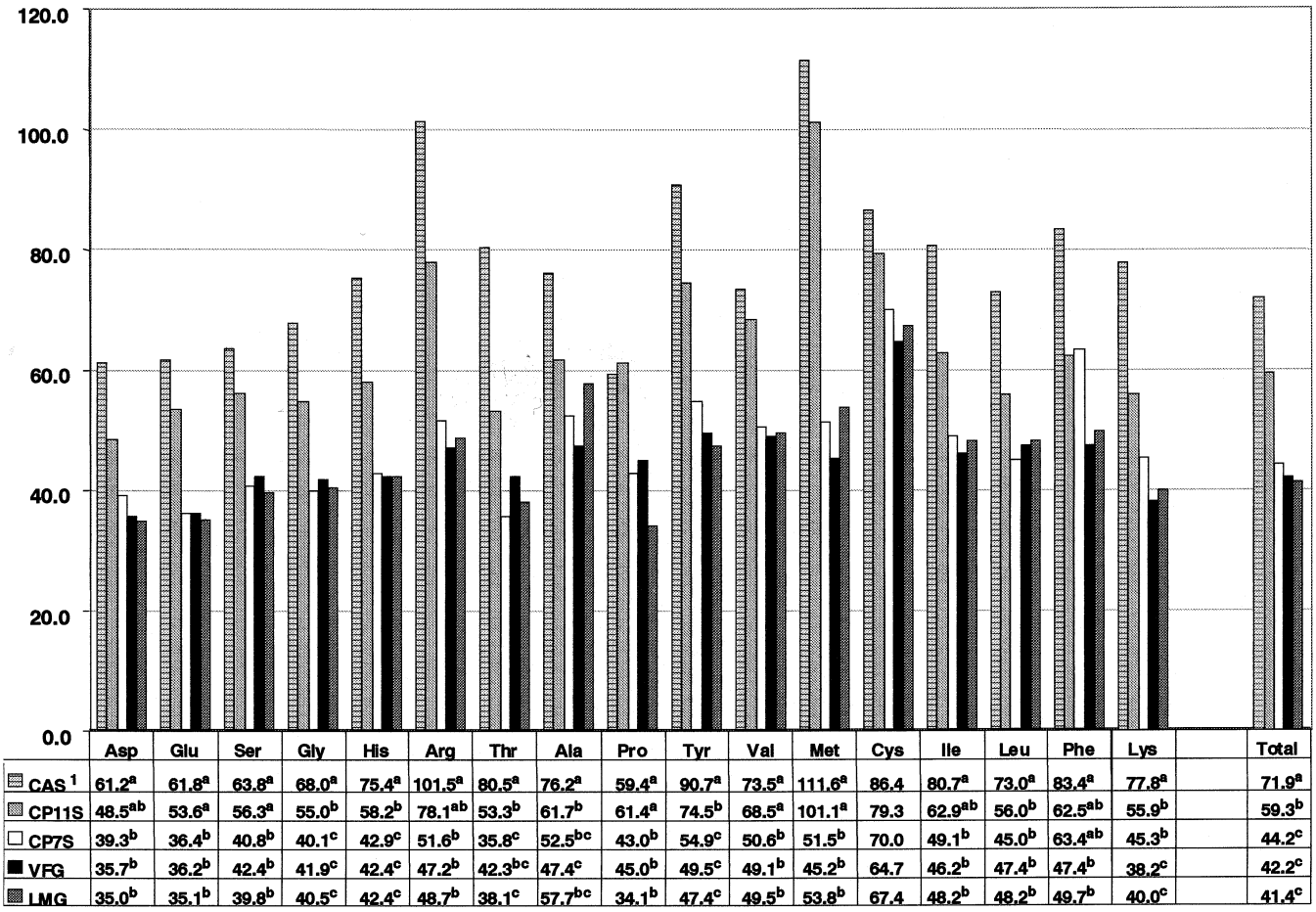
[a] CAS, casein; CP11S, chickpea 11S globulin proteins; CP7S, chickpea 7S globulin proteins; VFG, faba bean globulins; LMG, lupin meal globulins.

Table 3. Total Amounts of Amino Acids in Basolateral Chambers of Caco-2 Cell Cultures after 4 h of Exposure to Protein Digests (Micrograms per 2.5 mL)

	protein[a]					
	CAS	CP11S	CP7S	VFG	LMG	pooled SD
Asp	185.7a	339.7b	333.0b	369.2b	298.7b	48.5
Glu	641.6a	537.6ab	468.2b	584.4ab	670.6a	68.1
Ser	199.3	188.3	187.0	198.7	174.3	22.7
Gly	86.8a	131.2b	112.3ab	143.8b	135.4b	15.1
His	106.4	98.4	90.0	103.3	92.8	9.4
Arg	148.5a	350.8b	225.4ab	342.5b	375.2b	73.1
Thr	168.7	152.2	121.0	169.2	157.9	24.0
Ala	114.8a	149.5b	131.3ab	146.4b	118.1a	13.1
Pro	327.0a	198.7b	169.9b	202.9b	177.5b	30.9
Tyr	262.9a	137.7b	120.3b	152.6b	172.5b	16.5
Val	233.4a	178.1b	173.7b	190.6b	153.7b	29.4
Met	79.9a	55.8b	45.4b	35.7b	48.9b	14.3
Cys	29.3	27.9	27.1	23.5	28.6	6.0
Ile	140.8	114.6	113.3	124.4	116.2	26.6
Leu	307.4	242.4	281.2	307.7	277.6	40.7
Phe	201.1	220.0	235.6	182.4	161.6	34.9
Lys	320.6a	197.1b	211.4b	206.7b	145.0b	33.0
total	3554	3320	3046	3484	3304	341

[a] CAS, casein; CP11S, chickpea 11S globulin proteins; CP7S, chickpea 7S globulin proteins; VFG, faba bean globulins; LMG, lupin meal globulins. Means in the same row with different letters differ significantly ($P < 0.01$). Values are means of four different wells for each enzyme-digested protein.

were tested, only in vitro values have usually been reported. One of the few studies on in vivo digestibility with purified proteins is that by Aubry and Boucrot (*13*). They showed that after 2 h, gastric emptying and intestinal absorption of pea vicilin and legumin in rats were as high as those for casein. Also, more



	Asp	Glu	Ser	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Cys	Ile	Leu	Phe	Lys		Total
CAS [1]	61.2^{a}	61.8^{a}	63.8^{a}	68.0^{a}	75.4^{a}	101.5^{a}	80.5^{a}	76.2^{a}	59.4^{a}	90.7^{a}	73.5^{a}	111.6^{a}	86.4	80.7^{a}	73.0^{a}	83.4^{a}	77.8^{a}		71.9^{a}
CP11S	48.5^{ab}	53.6^{a}	56.3^{a}	55.0^{b}	58.2^{b}	78.1^{ab}	53.3^{b}	61.7^{b}	61.4^{a}	74.5^{b}	68.5^{a}	101.1^{a}	79.3	62.9^{ab}	56.0^{b}	62.5^{ab}	55.9^{b}		59.3^{b}
CP7S	39.3^{b}	36.4^{b}	40.8^{b}	40.1^{c}	42.9^{c}	51.6^{b}	35.8^{c}	52.5^{bc}	43.0^{b}	54.9^{c}	50.6^{b}	51.5^{b}	70.0	49.1^{b}	45.0^{b}	63.4^{ab}	45.3^{b}		44.2^{c}
VFG	35.7^{b}	36.2^{b}	42.4^{b}	41.9^{c}	42.4^{c}	47.2^{b}	42.3^{bc}	47.4^{c}	45.0^{b}	49.5^{c}	49.1^{b}	45.2^{b}	64.7	46.2^{b}	47.4^{b}	47.4^{b}	38.2^{c}		42.2^{c}
LMG	35.0^{b}	35.1^{b}	39.8^{b}	40.5^{c}	42.4^{c}	48.7^{b}	38.1^{c}	57.7^{bc}	34.1^{b}	47.4^{c}	49.5^{b}	53.8^{b}	67.4	48.2^{b}	48.2^{b}	49.7^{b}	40.0^{c}		41.4^{c}

Figure 2. Proportions of amino acids in basolateral chamber with respect to amounts added in the apical chamber of Caco-2 cell cultures after 4 h. CAS, casein; CP11S, chickpea 11S globulin proteins; CP7S, chickpea 7S globulin proteins; VFG, faba bean globulins; LMG, lupin meal globulins. $^{a-c}$Means in the same column with different superscript letters differ significantly ($P < 0.01$).

recently Carbonaro et al. (*14*) found that the in vivo digestibility of faba bean purified proteins was 86%, a value comparable to that previously determined and higher than that of lactalbumin (*15*). However, the nutritional value (measured as protein efficiency ratio and biological value) of diets based on purified legume proteins or even seed meals containing low or no amounts of ANF is below that of lactalbumin control diets even though ileal digestibilities of globulins purified from legume seeds such as soybeans (*Glycine max*), faba beans, (*Vicia faba*), and lupins (*Lupinus angustifolius*) were not different from control values in the rat (*16*−*18*). Furthermore, ileal and fecal N (*17*−*19)* or extracted protein (*15*) digestibilities of whole legume seed meals low in ANF were similar or close to control values. These results suggest that undenatured legume globulins are themselves highly digestible in the small intestine. Even though not all storage proteins are extracted in the conditions here used, buffer extraction yield is usually >80% of total protein in the seed meal (*16*−*18*). Therefore, the lower digestibility of legume proteins when the whole meal is used in the diet is more likely due to other factors such as lectins, tannins, and/or trypsin inhibitors. The results reported here add more to the same conclusion. Thus, as shown in **Figure 1**, proportions of amino acids in soluble fractions after in vitro enzyme digestion of legume buffer extracted proteins were not different from or even higher than those of casein, with the only exception being lysine in chickpea 11S and 7S. Kakade (*20*) reported that the primary structure of the proteins might explain to a great extent their digestibility due, for example, to the known specificity of trypsin for arginyl and lysyl peptide bonds. On the contrary, lysylprolyl and arginylprolyl bonds are resistant to trypsin. Thus, for a given protein, in theory the higher the ratio of arginine plus lysine to proline, the higher the rate of hydrolysis with trypsin. For the proteins studied here, that ratio ranged from 1.18 for casein to 2.97−4.45 for legume globulins. Therefore, as the present results also suggest, those proteins are likely to be readily digestible by digestive enzymes.

Changes in plasma amino acid concentrations have been previously reported in rats fed diets based on faba beans, lupin, chickpea, and soy meals or their respective globulin proteins (*21*). Thus, amounts of threonine, leucine, and lysine, the plasma values of which were substantially lower in rats fed equilibrated legume proteins, were similar or even higher in legume proteins compared to lactalbumin. Therefore, it seems that (a) as previously indicated by others (*22*) dietary concentration of amino acids on its own does not explain plasma differences and (b) plasma differences with legume proteins are likely to be more related with the absorption process in the intestinal mucosa than with the enzymatic hydrolysis of the proteins. Except for Asp, Gly, Arg, and Ala, amounts of amino acids transported from hydrolyzed legume proteins were not different from or even lower than casein controls (**Table 3**), even though amounts added in the apical chamber were usually higher (**Table 2**), due probably to higher in vitro digestibility values (**Figure 1**). As a consequence, as shown in **Figure 2**, the proportions of amino acids in the basolateral chambers of legume proteins after 4 h of incubation were lower for legume proteins than for casein. It seems therefore that the rate of transport from apical to basolateral chambers of amino acids might be slower for legume

proteins. That would agree with lower amounts of amino acids afferent per unit of time to the liver from the intestine recently determined in rats fed legume-based diets compared to controls (unpublished results). That might explain in turn the observed effects on liver and whole body N metabolism because the mix of amino acids available for protein synthesis in the tissues at a given moment could be unbalanced. This imbalance can lower the rate of protein synthesis, resulting in an increase in amino acid catabolism (*23*), lower protein retention values, and increased N excretion in the form of urea (*2*). As pointed out by Kies (*24*), it would appear that at least part of the explanation of the level of bioavailability of any specific protein must lie with its specific physical/chemical properties as determined by its amino acid content, amino acid sequence, and specific bonding. Those factors are likely to influence the rate of protein absorption and subsequently the efficiency of amino acid utilization for protein synthesis. Finally, Caco-2 cell cultures might become a useful model for studying amino acid absorption from different protein sources, replacing, to some extent, or in combination with animal trials.

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