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ABSTRACT   The effect of reducing the phytate in soy-protein isolates on nonheme-iron absorption was examined in 32 human subjects. Iron absorption was measured by using an extrinsic radioiron label in liquid-formula meals containing hydrolyzed corn starch, corn oil, and either egg white or one of a series of soy-protein isolates with different phytate contents. Iron absorption increased four- to fivefold when phytic acid was reduced from its native amount of 4.9–8.4 to < 0.01 mg/g of isolate. Even relatively small quantities of residual phytate were strongly inhibitory and phytic acid had to be reduced to < 0.3 mg/g of isolate (corresponding to < 10 mg phytic acid/meal) before a meaningful increase in iron absorption was observed. However, even after removal of virtually all the phytic acid, iron absorption from the soy-protein meal was still only half that of the egg white control. It is concluded that phytic acid is a major inhibitory factor of iron absorption in soy-protein isolates but that other factors contribute to the poor bioavailability of iron from these products.   *Am J Clin Nutr* 1992;56:573–8.

KEY WORDS   Soy-protein isolate, phytate, iron absorption

Introduction

Soy protein is a major ingredient in infant formulas especially in the United States where soy formulas now account for about one-quarter of infant-formula sales (1). The use of soy protein is also increasing in extended meat products, baked goods, and dairy-type foods. Good protein quality, low cost, plentiful supply, and excellent functional properties make it an attractive raw material for the development of new manufactured foods (2). One potential drawback to the use of soy protein is that it has an inhibitory effect on iron absorption in humans (3–7). Full-fat soy flour, textured soy flour, and isolated soy protein all markedly reduce nonheme-iron absorption. The isolated protein has the greatest inhibitory effect (3).

The nature of the substances in soybean products that inhibit iron absorption is unclear. However, soy-protein products are known to contain appreciable quantities of phytate, which is an important inhibitor of iron absorption in wheat bran (8).

The present study was designed to define the role of phytate in modifying nonheme-iron absorption from soybean-protein isolates in humans. A series of soy-protein isolates with a 1000-fold variation in phytate content were prepared. Nonheme-iron absorption from liquid-formula meals containing these soy-protein isolates was then measured in human volunteers with a radioisotopic method.

Materials and methods

Preparation of soybean-protein isolates

Eleven different soybean-protein isolates were prepared from three different batches of soy flour (**Table 1**). Isolates I–IV were standard isolates containing much of their native amount of phytic acid (4.9–8.4 mg/g of isolate). Isolates V–VII were low-phytate isolates (0.2–1.0 mg phytic acid/g) in which the phytic acid had been reduced by continuous acid-salt washing and ultrafiltration. In isolate VIII the phytic acid was reduced to < 0.01 mg/g by enzyme treatment. Isolate IX (< 0.01 mg phytic acid/g) was both enzyme treated and ultrafiltered. Finally, in isolates X and XI, phytic acid was restored to approximately its original amount by adding back sodium phytate to the low phytate isolates V and VIII, respectively. Isolates I and V were prepared from the first batch of soy flour and were fed in study 1. Isolates II, VI, and X were made from the second batch of soy flour and were fed in study 2. The remaining isolates were made from the third batch of soy flour and were fed in studies 3 and 4.

All isolates (≈1–2 kg) were prepared from commercial defatted soybean flour that was first soaked for 1 h in deionized water (flour to water ratio 1:7.5, wt:wt) and then centrifuged in a continuous system at 12000 × g at 37°C to remove the fibrous material. For the native phytate isolates (I–IV), the resulting soybean milk was adjusted to pH 5.2 to precipitate the protein, which was recovered by centrifugation, washed with deionized water, neutralized with potassium hydroxide, sterilized by steam injection at 140 °C, and spray-dried. The washing step removes some phytic acid and is the reason why our native phytate isolates contain 4.9–8.4 mg phytic acid/g compared with 9.0–17.0 mg/g in commercial isolates. Those isolates in which the phytate was removed by acid-salt washing and ultrafiltration (V–VII) were prepared in the same way except that after precipitation the protein fraction was ultrafiltered in a two-step process at pH 5.2 and pH 7 in the presence of sodium chloride (9).

For isolates in which the phytate was removed by enzyme treatment (VII and IX), the soybean milk was treated at pH 5.2 '

TABLE 1
Analytical data on soybean-protein isolates*

Soy-isolate fraction†	Percent crude protein	Phytic acid	Trypsin inhibitor	Iron	Calcium	Inorganic phosphorus
	%N × 6.25	mg/g	TIU/mg N	mg/g	mg/g	mg/g
Native phytate						
I[a]	96.4	8.4 ± 0.4‡	5.3	0.145	0.278	0.11
II[b]	87.7	7.2 ± 0	5.3	0.175	0.563	0.21
III[c]	89.1	6.5 ± 0.7	8.8	0.130	0.616	0.50
IV[c]	90.1	4.9 ± 1.1	6.7	0.140	0.462	0.09
Acid-salt–reduced phytate						
V[a]	92.0	0.2 ± 0	4.5	0.145	0.070	0.08
VI[b]	92.6	1.0 ± 0.1	3.6	0.180	0.440	0.19
VII[c]	90.1	0.3 ± 0.01	3.5	0.146	0.077	0.05
Enzyme-reduced phytate						
VIII[c]	89.7	≤ 0.01 ± 0.01	21.7	0.135	0.820	0.68
IX[c] §	91.8	≤ 0.01 ± 0	14.5	0.155	0.289	0.28
Restored phytate						
X[b] ‖	91.1	9.9 ± 0.2	7.2	0.162	0.364	0.17
XI[c] ¶	90.8	3.7 ± 0.2	8.8	0.145	0.142	0.22

* All analytical data were obtained on the spray-dried products without further moisture removal.
† Isolates with the same superscript were prepared from the same batch of soy flour.
‡ $\bar{x}$ ± SE.
§ Enzyme-reduced followed by ultrafiltration.
‖ Produced by adding sodium phytate to isolate VI.
¶ Produced by adding sodium phytate to isolate VIII.

with a phytase from *Aspergillus niger* (Alko Ltd, Helsinki, Finland) before precipitation of the protein. Isolate VII was then centrifuged, washed, neutralized, sterilized, and spray-dried as described above. For isolate IX the coagulum was subjected to an additional ultrafiltration treatment to remove the low-molecular-weight compounds. Isolates X and XI were made by adding sodium phytate back to the acid-salt–washed isolate V and the enzyme-treated isolate VIII, respectively, before the neutralization step.

Analytical methods

Iron and calcium were determined by atomic-absorption spectroscopy after dry ashing. Total nitrogen was determined by using an automatic nitrogen analyzer (Type NA 1500; Carlo-Erba, Milan, Italy). Trypsin inhibitors were measured according to the method of Kakade et al (10) and trypsin inhibitor units (TIU) were expressed per milligram nitrogen.

Phytic acid was measured by using a modification of the method of Makover (11) in which cerium replaced iron in the precipitation step. Phytic acid was calculated from the phosphorous content of the precipitate by using a factor of 3.55.

Inorganic phosphorous was extracted from 200 mg dried isolate with 10 mL 1 mol H_2SO_4/L. Phosphorous was measured immediately by using a microtitration plate assay based on the complex formation of malachite green with phosphomolybdate under acidic conditions (12).

Iron-absorption studies

Four iron-absorption studies were carried out in groups of 7–9 human subjects. In study 1 the volunteers were fed two test meals, each containing one of the experimental soy-protein iso-

lates, and a control meal containing egg white. Three test meals and a control meal were given in studies 2, 3, and 4. All test meals were fed as a semisynthetic liquid formula containing 67 g hydrolyzed corn starch (Fro-Dex, American Maize Products, Hammond, IN), 36 g corn oil (Nugget Brand, Stockton, CA), 12 mL vanilla extract (McCormick and Co, Baltimore, MD), 200 mL deionized distilled water, and 30 g protein (nitrogen × 6.25) derived from either a soy-protein isolate or egg white (Monarch Egg Corporation, Kansas City, MO). The calcium content of the soy-protein-isolate meals within each study was equilibrated by adding calcium chloride ($CaCl_2 \cdot 2H_2O$) to raise the calcium content to 44 mg/meal in study 1, 19.2 mg in study 2, and 27.4 mg in studies 3 and 4. The amount of calcium (96 mg/meal) in the egg white–control meal was not modified.

The test meals for studies 1–4 are described in **Table 2**. The test meals in study 1 included soy-protein isolate (I) containing its native phytic acid content and a low-phytate isolate produced by continuous acid-salt washing and ultrafiltration (V). Both isolates were prepared from the same soy flour. The test meals for the second study comprised a soy-protein isolate containing its native amount of phytic acid (II), a low-phytate isolate produced by continuous acid-salt washing and ultrafiltration (VI), and the same isolate to which phytic acid had been added back (X). Again, all isolates were prepared from the same soy flour. In the third study the test meals included a control soy-protein isolate containing its native amount of phytic acid (III), an isolate from which the phytate had been removed by enzyme digestion (VIII), and the same phytate-free isolate to which phytic acid had been added back (XI). For study 4 the meals were a control isolate containing its native phytic acid (IV), a low-phytate isolate produced by acid-salt washing and ultrafiltration (VII), and an

TABLE 2
Effect of phytate removal on iron absorption from soy isolates

Study, subjects, and mean age	Mean packed cell volume	Serum ferritin*	Meals†	Iron absorption*	Absorption ratio	
					vs meal A	vs meal D
	%	μg/L		% of dose		
1, (6 M, 2 F), 24 y	44	59 (49–71)	A Isolate I (native phytate)	1.50 (1.10–2.06)	—	0.24‡
			B Isolate V (A-S–reduced phytate)	3.15 (2.32–4.28)	2.10‡	0.50
			D Egg white control	6.34 (4.72–8.51)	—	—
2, (5 M, 4 F), 23 y	43	38 (29–50)	A Isolate II (native phytate)	0.92 (0.65–1.32)	—	0.16§
			B Isolate VI (A-S–reduced phytate)	1.91 (1.34–2.71)	2.07‡	0.33§
			C Isolate X (restored phytate)	1.08 (0.75–1.54)	1.17	0.19§
			D Egg white control	5.75 (3.96–8.33)	—	—
3, (7 M, 1 F), 23 y	45	68 (60–77)	A Isolate III (native phytate)	0.53 (0.41–0.68)	—	0.10§
			B Isolate VIII (E-reduced phytate)	2.50 (2.10–2.97)	4.75§	0.46‡
			C Isolate XI (restored phytate)	0.78 (0.52–1.15)	1.45	0.17§
			D Egg white control	5.48 (3.63–5.94)	—	—
4, (3 M, 4 F), 22 y	43	35 (28–45)	A Isolate IV (native phytate)	1.36 (0.94–1.98)	—	0.14§
			B Isolate VII (A-S–reduced phytate)	4.17 (3.01–5.76)	3.06§	0.43‖
			C Isolate IX (E-reduced phytate)	5.48 (4.16–7.21)	4.02‡	0.56
			D Egg white control	9.72 (7.56–12.51)	—	—

* Geometric $\bar{x}$ (±1 SE).
† A-S, acid-salt–reduced phytate; E, enzyme-reduced phytate.
‡ $P < 0.05$.
§ $P < 0.001$.
‖ $P < 0.01$.

isolate from which the phytate had been removed by enzyme treatment followed by ultrafiltration (IX). All isolates fed in studies 3 and 4 were produced from the same batch of soy flour. As indicated above, isolate IX differed from isolate VIII by having been subjected to an additional ultrafiltration step to remove low-molecular-weight compounds.

The volunteer subjects ranged in age from 20 to 31 y with a mean age of 23 y. There were 21 men and 11 women. They exhibited a wide range of iron status as reflected by serum ferritin concentrations between 11 and 138 μg/L. All were in good health and denied a history of disorders that are known to influence the gastrointestinal absorption of iron. Written, informed consent was obtained from each volunteer before beginning the investigation and all experimental procedures were approved by the Human Subjects Committee at the University of Kansas Medical Center.

Double sequential radioiron labels were used to measure iron absorption from four separate meals consumed by each subject. The meals were administered between 0700 and 0900 after an overnight fast and only water was allowed for the subsequent 3 h. All meals were labeled with the extrinsic label technique (13) by adding 37 kBq ^{59}FeCl$_3$ or 111 kBq ^{55}FeCl$_3$ to a solution of ^{56}FeCl$_3$ in 0.01 mol HCl/L containing a quantity of iron sufficient to adjust the total iron content of each meal to 5.7 mg in study 1, 6.4 mg in study 2, and 5.5 mg in studies 3 and 4.

On the day preceding the first test meal, 15 mL blood was drawn for the measurement of packed cell volume, serum ferritin (14), and background radioactivity. The first and second test meals were labeled with either ^{59}Fe or ^{55}Fe and administered on days 2 and 3 of the study. Blood (25 mL) was drawn on day 16 to measure incorporated red cell radioactivity. A similarly labeled

second pair of meals was given on days 16 and 17 in studies 2, 3, and 4. Only a single meal was fed on day 17 in study 1. The final blood sample was drawn 2 wk after the last test meal to measure the increase in circulating red cell radioactivity. Radioiron measurements were made on duplicate 10-mL samples of whole blood by a modification of the method of Eakins and Brown (15). Percentage absorption was calculated on the basis of the blood volume estimated from height and weight (16, 17) and an assumed red cell incorporation for absorbed radioactivity of 80% (18).

Percentage absorption values were converted to logarithms before statistical analysis and the results reconverted to antilogarithms to recover the original units (19). Because each study contained several independent manipulations of the test meals, paired t tests were used to compare absorption from selected test meals within the same study by determining whether the mean log absorption ratios differed significantly from zero.

Results

The results of the iron-absorption studies are shown in Table 2. In study 1, subjects fed the liquid-formula meal containing the control soy isolate (I) with a native phytic acid content of 8.4 mg/g had a mean iron absorption of 1.5%, which increased to 3.15% ($P = 0.01$) when the phytic acid content of the soy isolate was reduced to 0.2 mg/g by acid-salt washing and ultrafiltration (isolate V). In study 2 there was a similar twofold increase in iron absorption on feeding another low phytate isolate produced by acid-salt washing and, in addition, absorption returned to approximately its original amount when the phytic acid was added back. In this study, decreasing the phytic acid

from 7.2 (isolate II) to 1.0 mg/g (isolate VI) increased iron absorption from 0.92% to 1.91% ($P < 0.02$). Adding back phytic acid to an amount of 9.9 mg/g reduced iron absorption to 1.08%, which was not significantly different from its original value ($P > 0.2$).

A low phytate isolate produced by enzyme treatment was investigated in study 3. This isolate had a phytic acid content of ≤ 0.01 mg/g, compared with 0.2–1.0 mg/g in the low-phytate isolates produced by acid-salt washing and ultrafiltration (Table 1). In this study (Table 2), reducing the native phytic acid content of the control isolate (III) from 6.5 to ≤ 0.01 mg/g (isolate VIII) increased iron absorption almost fivefold from 0.53% to 2.50% ($P < 0.001$). Again, adding back phytic acid to 3.7 mg/g decreased iron absorption to 0.78%, which was not significantly different from its original value ($P > 0.2$).

Study 4 compared directly a low-phytate isolate produced by acid-salt washing (isolate VII) with a similar isolate produced by enzyme treatment (isolate IX). Both isolates were ultrafiltered to remove the low-molecular-weight compounds. Mean iron absorption from the control isolate (VI) containing 4.9 mg phytic acid/g was 1.36%. Reducing phytic acid to 0.3 mg/g by acid-salt washing increased absorption to 4.17% ($P < 0.001$) whereas reducing phytic acid to ≤ 0.01 mg/g by enzyme treatment increased absorption to 5.48% ($P < 0.05$). In this study there was no significant difference in iron absorption between these two low-phytate isolates ($P > 0.05$). However, when all the absorption ratios of the low-phytate isolates relative to their respective controls were combined (Fig 1), it is seen that acid-salt washing to produce isolates with 0.2–1.0 mg phytic acid/g increased iron absorption 2.3-fold, whereas enzyme treatment to give isolates with ≤ 0.01 mg/g phytic acid produced a significantly greater 4.4-fold increase ($P < 0.01$).

The mean iron absorption from the egg white–control meal was 6.34%, 5.75%, 5.48%, and 9.78%, respectively, in studies 1–4. As absorption from the egg white–control meal was measured in all subjects, it is possible to compare the absorption from different meals between studies by comparing their absorption
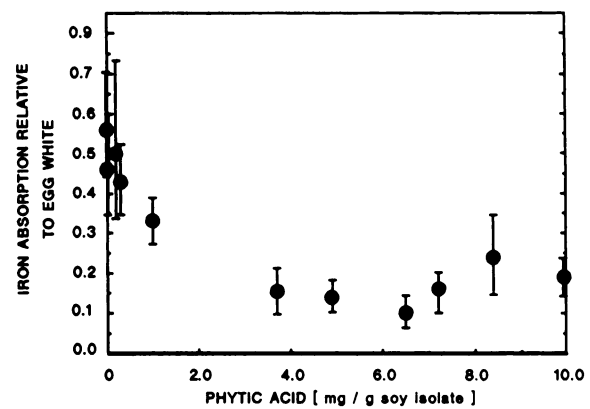
FIG 2. Relationship between phytate content (mg/g) of soy-protein isolate and mean iron absorption ± 1 SE of each soy-protein-isolate meal relative to its respective egg white control meal. (Data compiled from studies 1–4 as listed in Table 2). Approximate phytic acid content (mg) per meal can be calculated by multiplying the phytic acid content (mg/g) of the soy-protein isolates by 33.

relative to the egg white control. Thus, in **Figure 2**, iron absorption from meals containing the different soy-protein isolates relative to that from the egg white–control meal fed in the same subject (relative absorption = 1.0) is compared with the phytic acid content of the isolates. At phytic acid contents between 9.9 and 3.7 mg/g, iron absorption relative to the egg white control was low and varied randomly from 0.10 to 0.24. Only after phytic acid was reduced to ≤ 0.3 mg/g was there a substantial increase in iron absorption to 0.43–0.56 of the egg white control. Relative iron absorption can also be compared with the approximate phytic acid content of the meal. Because the hydrolyzed corn starch contained no measurable phytic acid, the soy-protein isolates were the only phytic acid–containing components of the meal. Each meal contained 30 g crude protein from the test isolate, and, because the protein contents of the isolates differed slightly (Table 1), is equivalent to ≈33 g isolate per meal. The phytic acid content per meal can be obtained by multiplying the phytic acid content of the isolate (mg/g) by 33. It can be seen that a substantial increase in iron absorption occurred only after phytic acid was reduced to < 10 mg/meal.

Discussion

The observation that consumption of wheat bran reduces iron absorption led Widdowson and McCance (20) to suspect that phytate may be an important inhibitor of nonheme food iron absorption. Subsequent investigations with bran have confirmed its inhibitory effect but other studies have yielded contradictory results as to the inhibitory nature of phytate specifically. In one study (21) the reduction of phytate in wheat bran was reported to have no effect on nonheme-iron absorption and monoferric phytate, which represents half the iron in wheat bran (22), was reported to be well absorbed. In other studies, the reduction of phytic acid in wheat bran did improve iron absorption (8) and adding phytic acid to wheat rolls inhibited iron absorption dose-dependently (23). The role of phytate in modifying nonheme-iron absorption from soy products was even more unclear as neither the removal of phytate from soy flour by acid washing (6) nor a twofold variation in the phytate content of soybeans
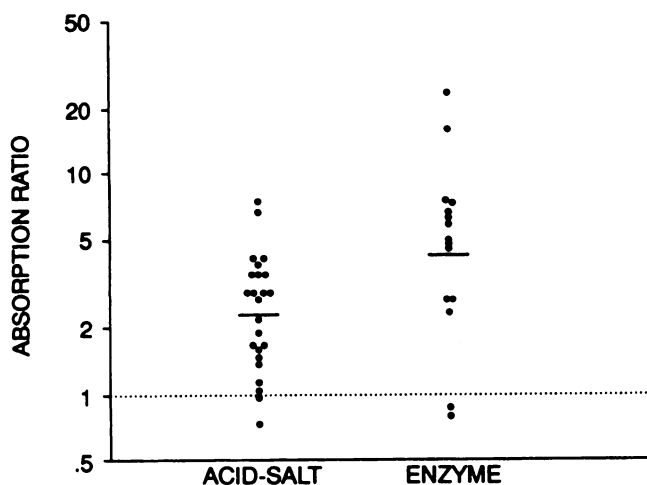

FIG 1. Iron-absorption ratios between reduced phytate soy-protein isolates and their corresponding control isolate containing its native phytic acid content. Acid-salt–reduced isolates contained 0.2–1.0 mg phytic acid/g and enzyme-reduced isolates contained ≤ 0.01 mg phytic acid/g. Short horizontal lines represent mean values.

produced under different growing conditions (24) influenced nonheme-iron absorption.

The present findings, however, now strongly suggest that phytic acid is a major inhibitory factor in soy-protein isolates. Removal of phytic acid to ≤ 0.01 mg/g of isolate increased iron absorption four- to fivefold whereas adding back the phytic acid reduced iron absorption to its original low value. Our results also demonstrate that relatively small amounts of phytic acid can still strongly inhibit iron absorption and that the phytic acid concentration in isolates must be reduced to ≥ 1.0 mg/g and optimally to < 0.3 mg/100 g to ensure a meaningful increase in iron absorption. The latter figure corresponds to < 10 mg phytic acid in a meal containing ≈5 mg Fe. The necessity for these very low amounts of phytic acid could explain why earlier studies failed to demonstrate a beneficial effect of reducing phytic acid in soy products (8, 24). By modifying the growing conditions, Beard et al (24) reduced the phytic acid content of soybeans from 7.04 to 3.76 mg/g. They fed the cooked beans as a soup or purée in meals providing ∼220 mg phytic acid in the high-phytate meal and 110 mg phytic acid in the low-phytate meal. They showed that reducing phytic acid by these amounts did not increase iron absorption relative to their reference meal. Our results would also suggest that decreasing the phytic acid content of a meal from 220 to 110 mg would have little effect on iron absorption but that by decreasing the phytic acid to < 10 mg/meal, iron absorption would be increased substantially.

Enzyme treatment was more effective at removing phytic acid than was acid-salt washing combined with ultrafiltration, giving isolates with ≤ 0.01 mg phytic acid/g compared with 0.2–1.0 mg/g. Acid washing with ultrafiltration, in addition to removing the phytic acid, removes a variety of low-molecular-weight compounds, which could also influence iron absorption. To investigate this possibility we subjected an enzyme-treated isolate to a further acid-salt washing with ultrafiltration. Our results would indicate that the ultrafiltration step did not further improve iron absorption. Absorption from the meal containing the enzyme-reduced phytate isolate VIII (Table 2) was 2.50% compared with 5.48% for the egg white–control meal. Absorption from soy isolate VIII relative to the egg white–control meal was thus 0.46. Absorption from the enzyme-reduced phytate isolate IX (Table 2) subjected to an additional ultrafiltration was 5.48% compared with 9.72% from the egg white–control meal in the same subjects. Absorption of soy isolate IX relative to the control meal was 0.56. The phytic acid content of both isolates was ≤ 0.01 mg/g.

Earlier studies comparing different protein sources incorporated into the same liquid-formula meals as administered in the present investigation demonstrated that soy-protein isolate was the most inhibitory of the protein sources tested. Iron absorption from a soy-protein-isolate meal with its native phytic acid content was 0.20 of the egg white–control meal (3) compared with 0.31 for wheat gluten, 0.40 for whey protein (25), 0.55 or 1.08 for casein (3, 25), 1.90 for bovine serum albumin (26), 3.00 for beef muscle (27), and 3.53 for the protein-free meal (26). In the present study, removal of virtually all the phytic acid from soy-protein isolates increased iron absorption from 0.10–0.24 to ≈0.55 of the absorption from the egg white–control meal, indicating that even after the removal of phytate, soy protein itself is still relatively inhibitory to iron absorption.

Earlier studies have shown that, at the same amount of ascorbic acid in the formula, iron absorption from milk-based infant formulas is two to three times higher than that from soy formulas

(28). Our results indicate that iron absorption from formulas made from phytate-free soy isolate would be similar to that from milk-based formula. Phytate-free soy-protein isolate, casein, and whey are still moderately inhibitory to iron absorption. The inhibitory nature of whey would appear to be due to its high calcium and phosphorous contents and not to its protein component (29). The inhibitory nature of casein and phytate-free soy isolate on the other hand is due probably to the binding of iron to insoluble peptides in the duodenum. The iron-binding peptides from casein are those that contain serine phosphate (29). The iron-binding peptides from soy could be those containing a high proportion of carboxylic acid groups. ▪

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