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Isolation and comparative characterization of α-amylase inhibitor from white kidney bean (*Phaseolus Vulgaris*): A serious *in vitro* assessment of the commercial product

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A B S T R A C T

Plant α-amylase inhibitors (αAIs) show great potential as tools to manipulate resistance of crop plants against pests. They can be also considered as drug-design target for treatment of diabetes and digestion disorder. In this study, an amylase inhibitor-rich fraction was purified by ethanol precipitation and affinity chromatography on chitosan beads column from white common bean (*Phaseolus vulgaris*) extract, and then its *in vitro* interaction with porcine pancreatic α-amylase (PPA) was studied. A commercially available amylase inhibitor, Phase 2, was also used to serve as a comparative reference. The results showed that inhibitory activity of the αAI extracted from white kidney bean was much higher than that of Phase 2. The purified inhibitor displayed significant heat stability, so that remaining inhibitory activity was ~80%, even at 60 °C for 30 min incubation. Fraction 3 retained ~84% of its initial activity after long term (45 days) dry storage at room temperature. Due to potency and appropriate heat/storage stability, this αAI preparation may be reconsidered as raw material for preparation of commercial αAI to control appetite and energy intake.

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Introduction

Alpha-amylase α-1,4-glucan-4-gllucanohydrolase, EC 3.2.1.1 and the protein inhibitor of α-amylase (αAI), which inhibits animal salivary and pancreatic α-amylase, have been identified and isolated from various plant/animal species [1]. αAI plays an important role in plant defense against pathogens and pests [2]. Amylase inhibitors are known as starch blockers because they prevent dietary starches from being digested and absorbed by the body. This could be useful for treating obesity and diabetes in human [3]. Despite relative progresses in the field [4], reaction mechanisms involved in the inhibition of human α-amylase by plant protein inhibitors are not completely understood [5]. But there is this possibility that reducing sugars which are covalently bound to the inhibitor polypeptide chain may play a major role in the mechanism or that the inhibitor may induce conformational changes in the enzyme molecule [6]. Among the plant sources, common bean (*Phaseolus vulgaris* L.) αAI has been reported to have relatively great potential as an extensive anti-obesity and anti-diabetes remedy, because it has not been associated with deleterious effects such as asthma and dermatitis which have been associated with some cereal αAIs [7]. Several companies have marketed αAI extracts from common beans for controlling appetite and energy intake [8]. Coloured common beans, particularly the large-seeded varieties, generally have a high level of phytohaemagglutinins (PHAs), which are considered as an anti-nutritional substance and may cause gastrointestinal disturbance [9]. Therefore, only the αAIs extracted from white common beans are used in commercially-produced αAI products [8],[10]. Many techniques that have been used to purify αAI from different plant species, such as salting out, ion exchange chromatography and gel filtration column chromatography [11], [12] are generally expensive and time consuming. However, searching for efficient and cheaper protocols for αAI preparation is of interest. Moreover, to the best of our knowledge, no post-marketing evaluation has been done on commercially available αAIs products in IRAN.

In the present work, αAI from white common beans (*Phaseolus vulgaris*) was isolated according to a new isolation procedure and its *in vitro* interaction with porcine pancreatic α-amylase (PPA) was studied. Inhibitory activity of obtained extract was compared with that of the commercial pharmaceutical product (phase 2). Also, additional characterization of the extract was carried out. In an attempt, thermal inactivation at different temperatures was studied and then storage stability, remaining biological activity after storage for a period of 45 days, was preliminary evaluated.

Materials and methods

Materials

All chemicals and reagents were of analytical grade. 3,5-Dinitrosalicylic acid (DNS) and soluble starch purchased from Sigma-Aldrich (St. Louis, MO, USA). Phase 2 (with commercial name of Carbo Blocker) α-amylase inhibitor was purchased from a local pharmacy. All other reagents used were of the highest grade commercially available. Each experimental point presented in the figures is average of at least (two or) three independent measurements with standard errors less than 5%. Whenever data were analyzed, $P < 0.05$ was considered statistically significant.

Purification of α-amylase inhibitor

White common beans were obtained from a local supermarket, and ground to a fine meal. Bean meal (500 g) was extracted with 5. Vol. of 10 mM sodium phosphate buffer (pH 7) with overhead stirring for 24 h at 4 °C. The extract was

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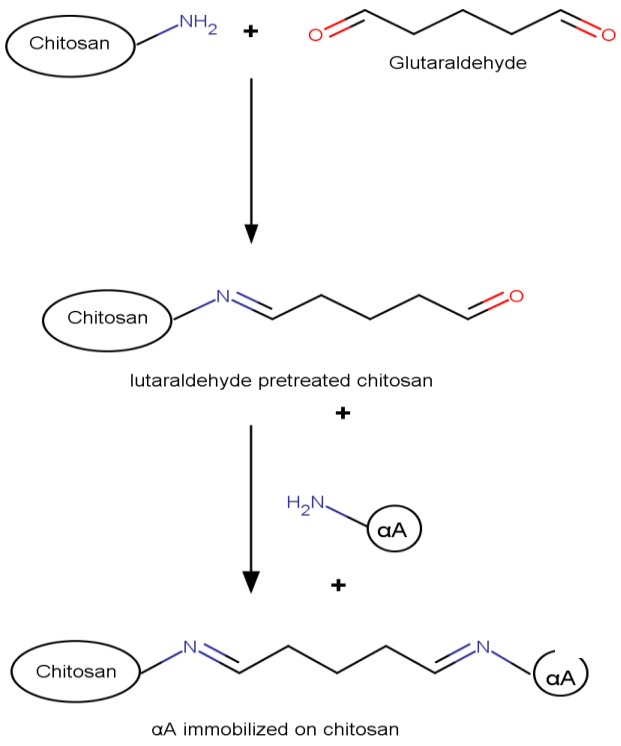
centrifuged at 8500×rpm for 30 min, filtered through glass wool, and made approx. 15% (v/v) in ethanol by slow addition of cold ethanol, with overhead stirring. After 30 min the mixture was centrifuged (5000× rpm for 1 h) (fraction 1), and more ethanol was added to the supernatant to a concentration of approx. 33% (v/v). The mixture was stirred for a further 30 min, and centrifuged (5000× rpm for 1 h) (fraction 2). At least, ethanol concentration in the supernatant was brought to 48% (v/v) and the mixture was centrifuged as before (fraction 3). Then, pellet was dissolved in 50 mM potassium phosphate buffer, pH 8, and loaded onto a chitosan beads column.

Preparation of chitosan beads

Chitosan beads (3%) were prepared in 1.5% (v/v) glacial acetic acid by heating at 50 °C. This solution was then added drop by drop into 150 ml 1 N KOH solution containing 25% (v/v) ethanol under stirring condition using a syringe needle. The solution was allowed to stand for 2 h at room temperature for hardening of chitosan beads. Beads of uniform shape and size obtained were immediately washed with double distilled water and stored in 20% ethanol at 4 °C until activation with glutaraldehyde [13].

Beads activation and α-amylase immobilization

Prepared chitosan beads were activated using 3% (v/v) glutaraldehyde at room temperature for 3 h. The activated beads were washed extensively with 50 mM potassium phosphate buffer (pH 8) to remove any free glutaraldehyde. The activated beads were incubated with 0.5 mg/ml porcine pancreatic α-amylase for 24 h at 4 °C for enzyme coupling (scheme 1). The beads were washed extensively with 50 mM potassium phosphate buffer to remove un-bound α-amylase. Chitosan beads were stored in 50 mM potassium phosphate buffer, pH 8.0, at 4°C, until used.



Scheme 1. Possible immobilization mechanism of α-amylase on glutaraldehyde pretreated chitosan [14].

Affinity chromatography

Bean amylase inhibitor(s) was purified via affinity chromatography as described by Pick et al [15]. Amylase inhibitor was allowed to bind the immobilized amylase at pH 5.6 (60 mM sodium acetate) and was eluted at pH 2.8, using 50 mM glycine-HCl buffer [16].

α-Amylase inhibitory activity

The α-amylase inhibitory activity was assayed by measuring the residual α-amylase activity in the presence of the sample extract containing the inhibitor. The assay was performed by adding from 4 to 32 µL of sample extract to 20 µl of

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porcine pancreatic α-amylase solution, to obtain 50% inhibition. An appropriate blank was prepared without α-amylase in order to correct for any endogenous amylase activity. The mixtures were brought to a total volume of 200 µL with 20 mM phosphate buffer pH 6.9 containing 6.7 mM sodium chloride, then samples were pre-incubated at 37 °C for 30 min [17]. After the addition of substrate solution (1% soluble starch) and incubation for 5 min, the reaction was stopped by the adding of 400 µl of 3,5 dinitrosalicylic acid reagent, followed by boiling for 5 min in a water bath. Then 5 ml of water was added and the solution was mixed and allowed to stand at room temperature for 15 min. Absorbance was measured at 545 nm.

α- Amylase inhibitory activity after dry storage

Fraction 3 was prepared (as stated earlier), placed within oven and dried at 35 °C for 2 days, then to assess storage stability of the inhibitor preparation, it was transferred to a sealed glassware and incubated at room temperature (25 °C) for 45 days, and then α-amylase inhibitory activity of fraction 3 was measured at 1st, 14th and 45th days of incubation, as it has been previously described.

Thermal inactivation

Irreversible thermo-inactivation of the αAI was investigated at different temperatures ranging from 30 to 70 °C, for 5-30 min. At regular intervals, sample were removed and immediately cooled on ice. Thereafter, the residual inhibitory activity was measured by adding 20 µL of the enzyme (20 µg/ml) to 180 µL of αAI solution and pre-incubated at 37 °C for 30 min then substrate was added to the mixture. The activity of the enzyme solution in the absence of αAI was considered as the control (100%) [18].

Results and discussion

Aqueous extract preparation/α-amylase inhibitor purification

The first step in the current study was purification of α-amylase inhibitor from white common beans. A crude inhibitor preparation from common bean seeds was obtained by aqueous extraction and a three-step fractionation by ethanol. This gave a partially purified αAI that inhibit porcine pancreatic α-amylase (data not shown). Then the inhibitor was enriched/purified from the last ethanol fractionation (fraction 3) by affinity chromatography using porcine pancreatic α-amylase coupled to chitosan beads. The absorbed proteins by the affinity beads were analyzed by SDS-PAGE (Fig. 1). According to the literature, the αAIs are tetramer (α2β2) glycoproteins with molecular weight ranged from 36 to 56 kDa [19] which composed of 15 to 18 kDa subunits [20]. Thus, the αAI might be dissociated into relatively smaller peptides during electrophoresis. In this study, the isolated αAI (Fig. 1 lane B) contained two peptide fractions with the molecular weight ranged between 14 and 18 kDa, together with another two fractions around 27 to 32 kDa. The peptide fractions ranging between 14 and 18 kDa corresponded to α and β subunits. The larger proteins (between 27 and 32 kDa) probably corresponded to the unprocessed αAI proproteins as suggested by Pueyo et al. (1993) [21] or undissociated aggregates of the smaller polypeptides [22]. This profile is also similar to the αAI profiles for a white common bean variety reported by Tormo et al. (2006) and Wang et al. (2011) [19,23].

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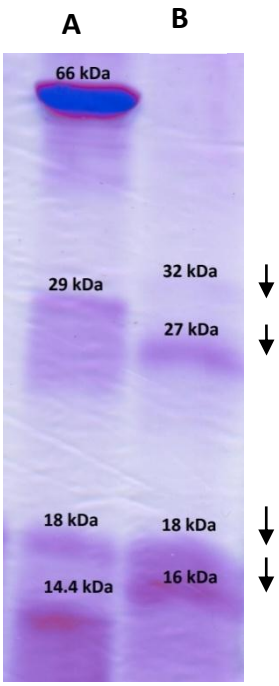

Fig. 1. Polypeptide pattern of α-amylase inhibitor under SDS-PAGE separation (12.5% slab gel) and Coomassie brilliant blue staining: Lane A: molecular weight of markers, lane B: αAI (two peptide fractions with the molecular weight around 16 and 18 kDa, with another two peptide fractions around 27 and 32 kDa) purified after three-step fractionation using ethanol followed by affinity chromatography.

Inhibitory activity of fractions/purified αAI

The inhibitory activities of three isolated fractions of white common bean extract (15%, 33% and 48% ethanol) against porcine pancreatic α-amylase were determined *in vitro* (Fig. 2). The α-amylase inhibitory activity was assayed by measuring the residual α-amylase activity after the enzyme and inhibitor were pre-incubated for 30 min at 37 °C. As reported previously [24], no immediate inhibition was observed when substrate, PPA and αAI were mixed together. The incubation period ensures that complete equilibrium is reached between the enzyme, the inhibitor and the enzyme-inhibitor complex and allows us to postulate that the system is at equilibrium. As shown in figure 2, all of fractions showed inhibitory activity. It was found that the α-amylase inhibitory activity increased in the order of fraction 3 (93.2%) > fraction 2 (51.1%) > fraction 1 (37.4%). Fraction 3 showed the much higher amylase inhibitory activity and thus was selected for further investigation.

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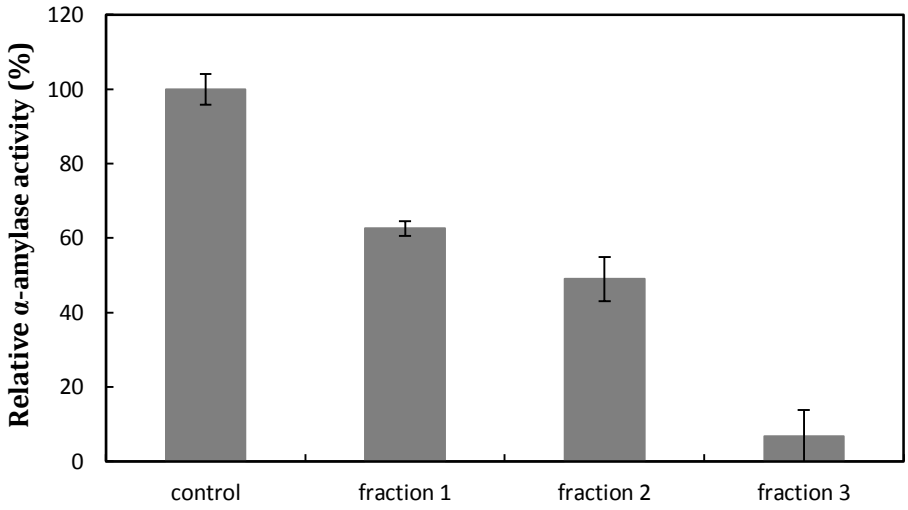

Fig. 2. Amylase inhibitory activities of three isolated fractions of white common bean extract (fractions 1, 2 and 3 correspond to 15%, 33% and 48% ethanol, respectively) at the same concentration against porcine pancreatic α-amylase. Both the enzyme and inhibitor were preincubated for 30 min at 37 °C. The activity of the enzyme solution in the absence of αAI was considered as the control (100%).

Additional characterization of the inhibitory activity of fraction 3 against the porcine pancreatic α-amylase was performed. Inhibition of the α-amylase by fraction 3 was concentration-dependent as shown in Fig. 3. As the concentration of αAI protein increased, the residual α-amylase activity was decreased. Similar results were also reported by Lee Berre-Anton et al. (1997) [24]. The enzyme inhibition strength is usually expressed as the IC_{50} value, which is the concentration of an inhibitor needed to inhibit half of the enzyme activity, in the tested condition. Additionally, we used a commercially available product, phase 2 (with trade name of Carbo Blocker) α-amylase inhibitor to serve as a comparative reference. To ensure the dissolution of the protein content of commercial product, several buffer systems were tested. Surprisingly, the phase 2 showed no detectable amylase inhibitory activity in the protein concentration range from 0.04 to 0.32 mg/ml. Additionally, IC_{50} value for fraction 3 was estimated as IC_{50}=0.107 mg/ml. Our results showed that the fraction 3, extracted from white kidney beans, was highly active against porcine pancreatic α-amylase, and completely inhibited starch hydrolysis with addition of 0.2 mg of αAI protein. Therefore, fraction 3 was recognized as a more effective inhibitor than commercial product, phase 2.

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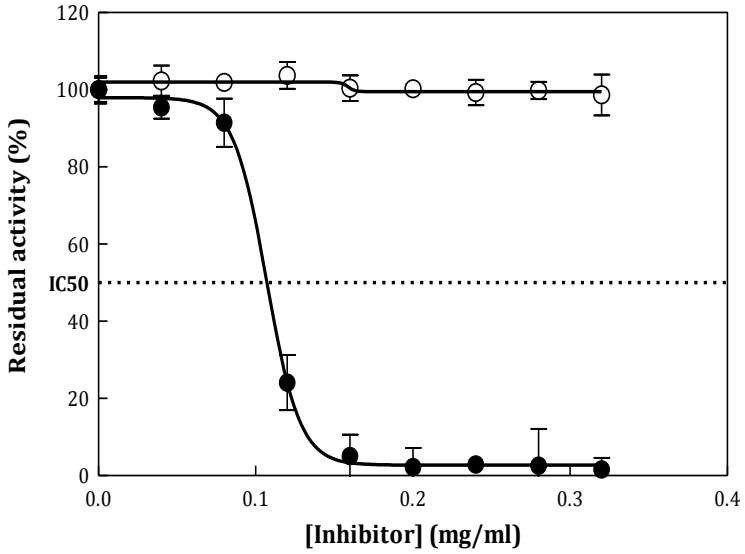

Fig. 3. Comparison of inhibitory effect of fraction 3 (●) and phase 2 (○) on the activity of porcine pancreatic α-amylase (PPA). The phase 2 show no inhibitory activity against PPA at the concentration range from 0 to 0.32 mg/ml but fraction 3 was potent αAI (IC_{50}=0.107 mg/ml).

Residual αAI activity after storage

Purified proteins often need to be stored for an extended period of time while retaining their original structural integrity and/or activity. The extent of storage "shelf life" can vary from a few days to more than a year and is dependent on the nature of the protein, impurity and the used storage conditions. Optimal conditions for storage are distinctive to each protein. Hence, the fraction 3 was incubated at room temperature (25 °C) for 45 days. The inhibitory activity of fraction 3 was measured in the initial, 14th and end of the incubation period. After two weeks, αAI activity was decreased 4% compared to the first day of incubation, while its activity was decreased as much as 14% at the end of incubation (Fig. 4). It is noteworthy that this is only a preliminary assessment and protein pharmaceutical formulations must pass other standard tests for physic-chemical stability.

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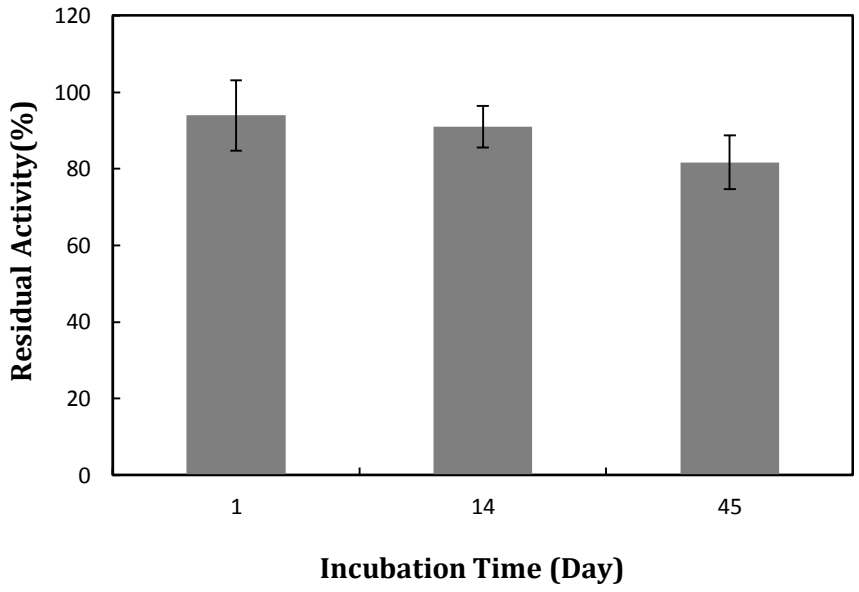

Fig. 4. The α-amylase inhibitory activity of fraction 3 after 45 days incubation at room temperature (at 25°C). Fraction 3 was dried in 35 °C for 2 days and then incubated. At 14th day, αAI activity was decreased 4% compared to that of the first day, while its activity was decreased 14% after 45 days of incubation.

Thermal stability of the αAI

Biological activity and clinical efficacy of a therapeutic protein are contingent upon the structural stability, bioavailability, and clearance rates of the protein. Temperature is one of the most important parameters that affect protein stability (half-life). Prathibha et al. (1995) [25] and Ali et al. (2012) [26] reported that α-amylase inhibitors are fairly heat-stable. The irreversible thermo-inactivation of the fraction 3 (as partially purified αAI) was investigated at different temperatures between 30 and 70˚C. Fig. 5 shows the influence of temperature on the αAI biological activity. Thermo-stability profile indicated that the inhibitor was almost stable in a temperature range from 30 °C to 60 °C but the amylase inhibitory property declines sharply at 70 °C. As indicated by the irreversible thermo-inactivation analyses, αAI retains only ~20% of its initial activity after 30 min of incubation at 70 °C.

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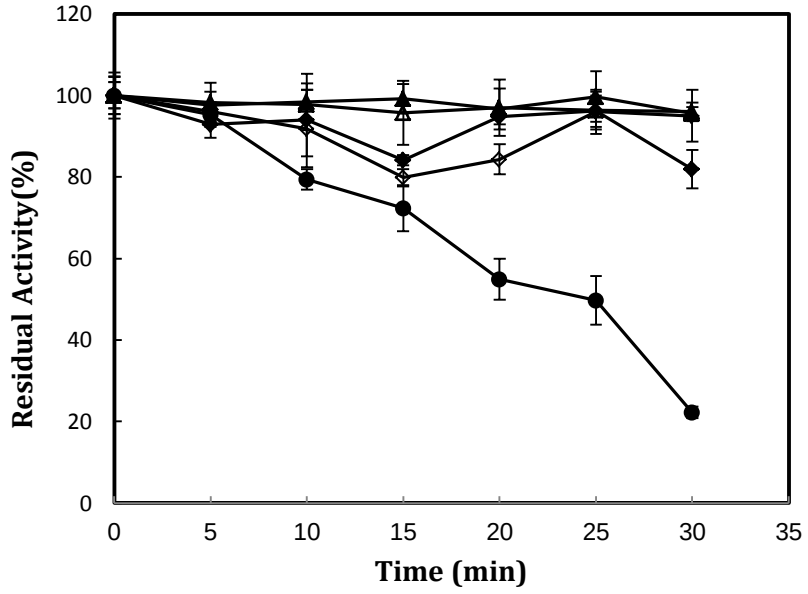

Fig. 5. Irreversible thermal inactivation of αAI at 30 °C (♦), 40 °C (◊), 50 °C (▲), 60 °C (△) and 70 °C (●). The activity of the enzyme solution in the absence of αAI was considered as the control (100%).

Conclusion

The high inhibitory activity of fraction 3 showed that the ethanol fractionation and affinity chromatography are the proper techniques to (partially) purify the αAI from *P. vulgaris* seed. The results indicated that the αAI extracted from white kidney bean had considerably greater amylase inhibitory potency (IC_{50}=0.107 mg/ml) than that of Phase 2 commercial product. Also, this heat-stable protein was stable up to 60 °C and retained its biological activity more than 80% after 45 days incubation at room temperature. Thus, it seems that the fraction 3 can be considered as raw material for pharmaceutical preparation of αAI to control appetite and energy intake, after successful optimizing formulation conditions to increase half-life of the product.

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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