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Research Article

OPTIMAL EXTRACTION PROCESS AND IDENTIFICATION OF A-AMYLASE INHIBITORS FROM *POUTERIA SAPOTA*

T. SATHISHKUMAR*, S. ABARNA, M. MALINI, S. NITHYA, P. PRATHISHTA AND J. LAVANYA

Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, India. E.mail: sathishkumart29@gmail.com

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ABSTRACT

The aim of the present study was to optimize the extraction process of polyphenolic pancreatic α- amylase inhibitors from the kernel of *Pouteria sapota* was optimized using L_{16} orthogonal design of experiment. The effect of single factors such as centrifugal speed, centrifugation time, isolation buffer pH and solid-liquid ratio on the extraction of the α-amylase inhibitors were also investigated. The results proved that about 95.8% pancreatic α-amylase inhibitory activity was obtained under optimum conditions of centrifugal force at 4000 rpm for 5 minutes with a material ratio of 1:20 with the isolation buffer pH 7.5. The TLC analysis revealed the presence of rutin and quercetin related flavonoids in the kernel.

Keywords: α-amylase inhibitors, Diabetes mellitus, Flavonoids, Orthogonal design, *Pouteria sapota*

INTRODUCTION

Diabetes mellitus and cardiovascular diseases are challenging medical and social problems. Individuals with diabetes mellitus are at a higher risk of developing vascular dysfunction and hypertension[1]. It is improperly regulated by the pancreatic hormone, insulin resulting in an increased blood glucose level. Type 1 diabetes also known as insulin dependent diabetes mellitus (IDDM) is an autoimmune disease where, the body's immune system attack and necrose the β- cells of pancreas that secretes insulin. Type 2 diabetes (Non insulin dependent diabetes mellitus, NIDDM) is caused due to insulin resistance that leads to a decreased insulin efficiency to induce glucose transport from blood to key target cells. Global prevalence of diabetes is estimated to be 366 million by 2030[2].

α-amylase inhibitors play a major role in managing postprandial hyperglycemia (PPHG) in diabetic patients. They inhibit the action of α-amylase, an enzyme that catalyzes the endohydrolysis of α (1→ 4) glucosidic linkage present in starch and thereby, leading to a reduction in starch hydrolysis[3]. This kind of inhibition shows beneficial effects on glycemic index control in diabetic patients. The chemical nature of amylase inhibitors may be proteinase or non proteinase (polyphenols). Generally, proteinase inhibitors are widely distributed among cereals and legumes. The favoured hypotheses about physiological roles of proteinase enzyme inhibitors in seeds are that they act as storage or reserve proteins as regulators of endogenous enzyme or as defensive agents against the attacks of animal predators and insect or microbial pests[4]. Low molecular weight plant-derived molecules such as polyphenols, luteolin, myricetin, and quercetin were potent inhibitors against porcine pancreatic α-amylase and the potency of inhibition correlated with the number of hydroxyl groups on the B ring of the flavonoid scaffold[5]. α-amylase and its inhibitors is drug-design targets for the development of compounds to treat diabetes, obesity and hyperlipaemia[4]. These inhibitors show remarkable structural variety leading to different modes of inhibition.

Herbs have recently attracted attention as health beneficial foods and as source materials for drug development[6]. Many plants are known for controlling diabetes but only few have been evaluated and principles have been isolated. The demands of tropical and subtropical fruits have been increased in the last two decades due to its good sensorial characteristics and high nutrimental value. Particularly, Sapote (*Pouteria sapota*) has been considered to have great economic potential because of its typical taste and aroma[7]. *Pouteria sapota* (Sapota) is an alternative commercial crop for tropical and subtropical regions of the world[8]. It has been used ethnobotanically to treat diarrhea, stomach ache and dysentery[9]. The kernel of *Pouteria sapota* yields 45-60% oil that was used in preparation of shampoo cosmetics and other pharmaceutical products. The oil is also used to treat sinusitis, asthma and epilepsy. The residue after oil extraction is applied to painful skin diseases. Prediction of separation conditions are not yet straight forward and in this case orthogonal design of experiment can be applied to find the optimal conditions for the extraction of polyphenolic inhibitors and the effect of the main variables in the extraction can be also investigated[10].

At present, there are no scientific documentation on the optimal extraction process of α-amylase inhibitors from the seed kernel of *Pouteria sapota.* So, in our laboratory, we have focused our attention to explore the optimal conditions that extract the polyphenolic α-amylase inhibitors and their possible utilization in the area of healthcare.

MATERIALS AND METHODS

Porcine pancreatic α-amylase (Sigma – Aldrich 3176), Acarbose (Bayer Pharmaceuticals private Limited, India), Quercetin (SD Fine chemicals Ltd, India), Rutin (SD Fine chemicals Ltd, India), 3, 5-dinitrosalicylic acid, silica gel G60 for TLC (Merck chemicals India), Beckman DU 530 UV/Vis spectrophotometer. *Pouteria sapota* was purchased from the local market and its kernels were used for experimental analysis. All other chemicals and solvents used in the experimental analysis were of analytical grade.

Extraction parameters

Factors like centrifugal speed, centrifugation time, pH and solid-liquid ratio were analyzed individually for the extraction of α-amylase inhibitors from the dried kernel material. L_{16} orthogonal design of experiments (i.e.,) four levels with four different parameters was used to optimize the extraction conditions (table 1 and 2). A single factor analysis of variance (One way ANOVA) was adopted to investigate the effect of each factor in the extraction of polyphenolic α-amylase inhibitors using Sigmastat 3.5 trial version software.

Table 1: Factors for the extraction of α-amylase inhibitors

	A	B	C	D
Level	**pH**	**Time (minutes)**	**Sol:Liq (g/ml)**	**RPM**
1	6	5	01:05	3000
2	6.5	10	01:10	4000
3	7	15	01:15	5000
4	7.5	20	01:20	6000

Table 2: L_{16} orthogonal design of experiment

Exp	A	B	C	D
1	1	1	2	3
2	1	2	1	4
3	1	3	4	1
4	1	4	3	2
5	2	1	1	1
6	2	2	2	2
7	2	3	3	3
8	2	4	4	4
9	3	1	3	4
10	3	2	4	3
11	3	3	1	2
12	3	4	2	1
13	4	1	4	2
14	4	2	3	1
15	4	3	1	4
16	4	4	2	3

Estimation of α-amylase inhibitory activity

α-amylase inhibitory activity of the extract was analyzed by the method of Bernfeld[11] with a little modification as explained below. To 100 µl of test extract, 200 µl porcine pancreatic α-amylase enzyme (Sigma – Aldrich 3176, 1 mg/ 10 ml phosphate buffer, pH 6.9) and 100 µl of 2 mM of phosphate buffer (pH 6.9) was added. After 20 min incubation, 100 µl of 1% starch solution was added. The same was performed for the control where 100 µl of test extract was replaced by buffer. After 5 min incubation 500 µl of 3,5-dinitrosalicylic acid reagent was added to both control and test. The tubes were kept in a boiling water bath for 5 min. The absorbance was recorded at 540 nm using Beckman DU 530 UV/Vis spectrophotometer and the percentage inhibition of α amylase enzyme was calculated using the formula:

Inhibition (%) = 100 x (control - test) / control)

Identification of flavonoids by Thin Layer Chromatography (TLC)

The optimal extract was run in one dimensional chromatography in the mobile phase using solvent (ethyl acetate ethanol-water, 5:1:5, v/v/v) at room temperature of 20-25°C. The concentrated extracts were spotted on the lower left of the TLC plate and the diameter of the spot in each chromatogram was normally about 1.5 cm. Flavonoids were identified under UV light after application of ammonia.

RESULTS

Fig. 1 showed that centrifugal force of 4000 rpm was found to be optimum for the extraction of α-amylase inhibitors. The optimal centrifugal time for the extraction of α-amylase inhibitors was found to be of 5 min (fig. 2).

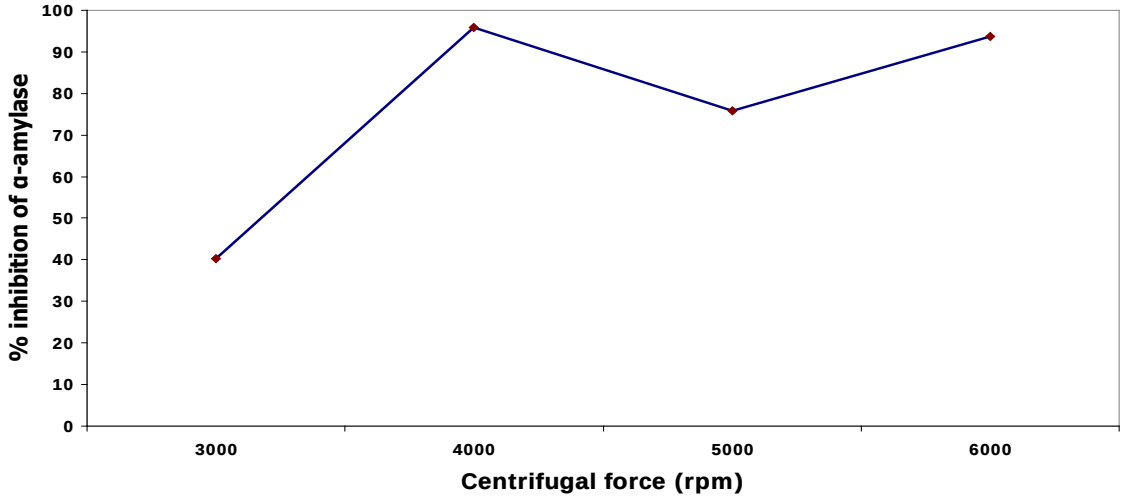

Fig. 1: Effect of Centrifugal force on extraction of α-amylase inhibitor

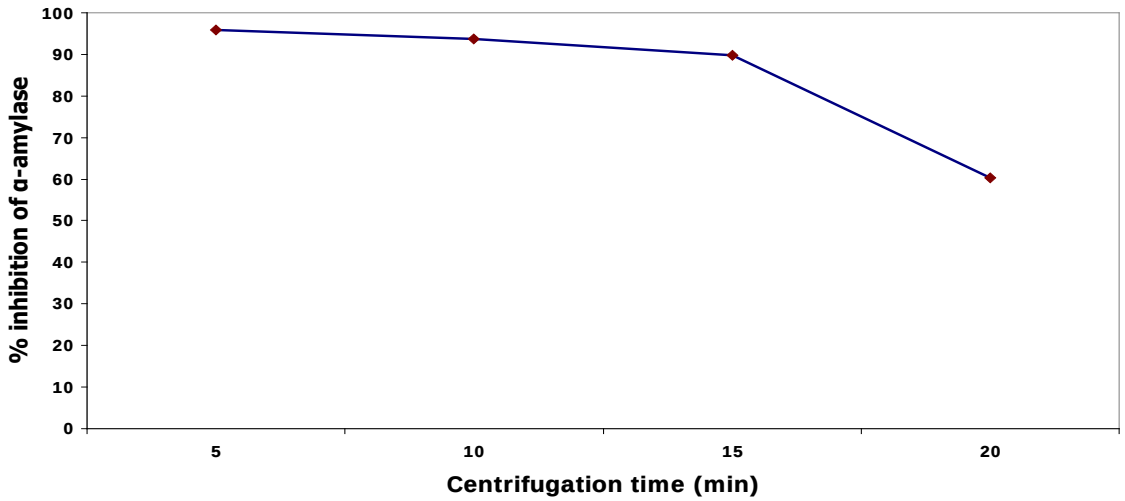

Fig. 2: Effect of Centrifugal time on extraction of α-amylase inhibitor

From fig. 3, the optimal solid –liquid ratio for the extraction of α-amylase inhibitors was found to be 1:20. The result also showed that the extraction yield of α-amylase inhibitors decreased significantly with the ratio of solvent to raw material (solutes) in a range of 5 to 15 and changed significantly when the ratio was greater than 15. The pH 7.5 was found to be optimum for the extraction of α-amylase inhibitors (fig. 4).

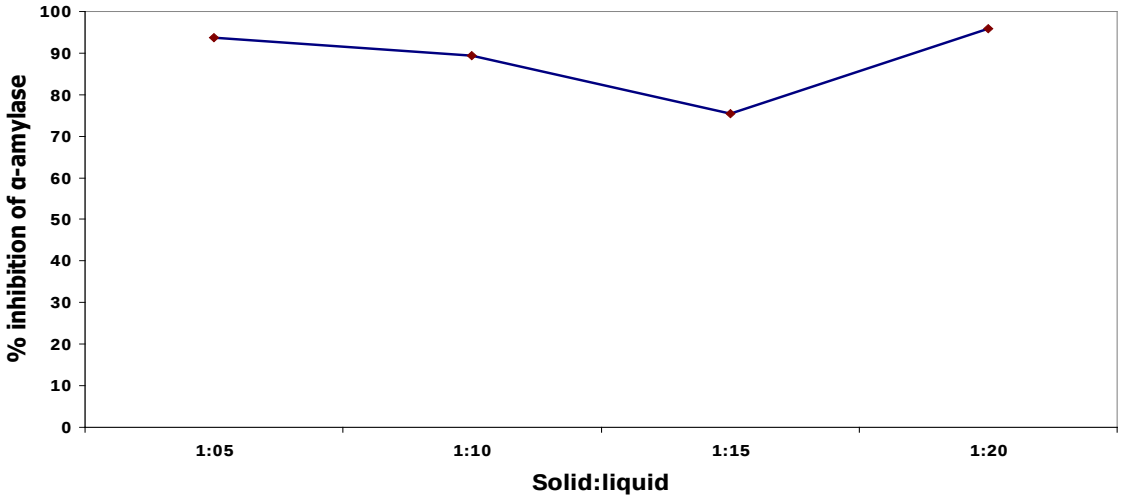

Fig. 3: Effect of Solid:liquid on extraction of α-amylase inhibitor

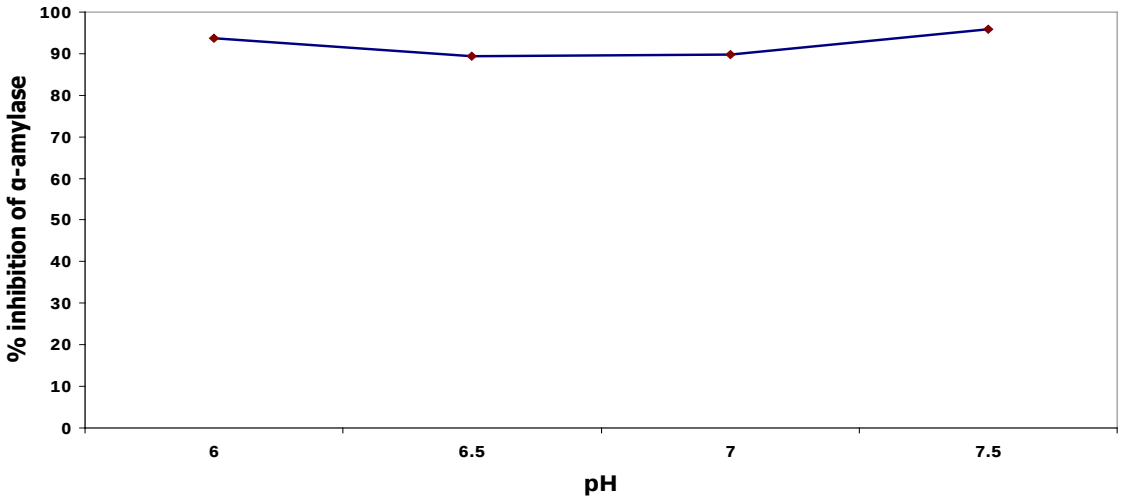

Fig. 4: Effect of pH on extraction of α-amylase inhibitor

Under the above specified optimum conditions the maximum pancreatic α-amylase inhibitory activity was observed as 95.8% (table 3 and table 4).

Table 3: one way ANOVA analysis

Levels	Sum of squares	df	Mean square	F
A	342.29	3	114.09	0.08
B	2194.08	3	731.36	0.644
C	1531.67	3	510.56	0.429
D	9277.89	3	3092.63	5.69
		12		

Sigmastat 3.5 software was used to analyze the range and one way ANOVA for the results obtained from the orthogonal design of experiment. D > B > C > A was the order of the effect of factors on pancreatic α-amylase inhibitors extraction. Centrifugal speed (rpm) had the greatest effect on the extraction procedure and it was found to be significantly different at 1 % level ($p < 0.01$). An equivalent effect was observed in centrifugation time, pH of isolation buffer and solid-liquid ratio in the extraction of α-amylase inhibitors. However, these factors did not play a vital role in the extraction of α-amylase inhibitors to a higher yield and was also found to be not significantly different at both 1% ($p > 0.01$) and 5 % ($p > 0.05$) level. The optimum extraction conditions obtained from the statistical analysis was found to be A_4 (7.5 pH) B_1 (5 min) C_4 (01:20) D_2 (4000 rpm). The results of TLC have revealed the presence of rutin and quercetin related flavonoids that may act as potent α-amylase inhibitors (fig. 5).

Table 4: experimental result and range analysis

Exp	A	B	C	D	α-amylase inhibitor %
1	1	1	2	3	53.43
2	1	2	1	4	93.77
3	1	3	4	1	40.3
4	1	4	3	2	51.9
5	2	1	1	1	11.7
6	2	2	2	2	89.4
7	2	3	3	3	75.4
8	2	4	4	4	21
9	3	1	3	4	9.45
10	3	2	4	3	75.9
11	3	3	1	2	89.7
12	3	4	2	1	19.4
13	**4**	**1**	**4**	**2**	**95.8**
14	4	2	3	1	3.4
15	4	3	1	4	3.9
16	4	4	2	3	60.3
K_1	59.85	42.595	58.54	18.7	
K_2	49.375	65.62	55.63	81.7	
K_3	48.61	61.1	35.03	66.26	
K_4	49.625	38.15	58.25	40.81	
k_1	14.96	10.64	14.635	4.67	
k_2	12.34	16.4	11.13	20.43	
k_3	12.15	15.28	8.76	16.57	
k_4	12.4	9.54	14.56	13.2	
R	2.81	6.86	5.86	15.9	

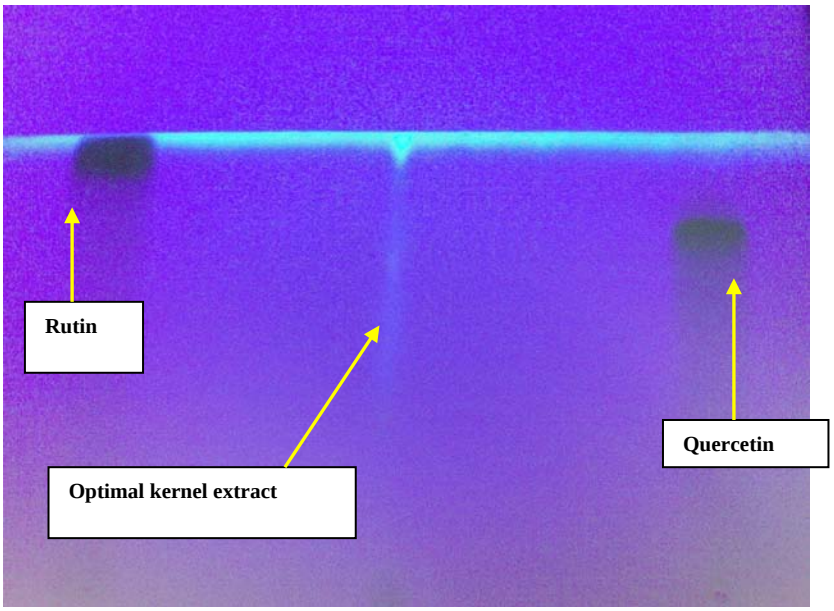

Fig. 5: Identification of flavonoids from optimized extract by TLC under far UV light

DISCUSSION

Enzyme inhibitors are important tools of nature for regulating the activities of enzymes in case of emergency. Plant seeds are known to produce a variety of enzyme inhibitors that are thought to protect the seed against insects and microbial pathogens[12]. Proteins that inhibit α-amylases are found throughout the plant kingdom. Many of the abundant proteins in cereal seeds are inhibitors of either *α*-amylase or protease inhibitors (PIs), or both. The *α*-amylase inhibitory activities of these proteins are usually directed against α-amylases from animals including a broad spectrum of insects and micro-organisms, but rarely against plants amylases[13]. Similarly, among flavonoids, six different groups including anthocyanidin, isoflavone and flavonols were proved to possess significant inhibitory activity against yeast and rat small intestinal α-glucosidases and porcine pancreatic α-amylases.

The enhanced inhibitory activity was, may be due to the presence of the unsaturated C ring, 3-OH, 4-CO, the linkage of the B ring at the 3 position and the hydroxyl substitution on the B ring in the flavonoids. Luteolin, myricetin and quercetin were proved to be the potent inhibitors for porcine pancreatic α-amylase[14]. In general, a full evaluation of the effect of four different parameters at four levels on the yield would require 256 (4^4) experiments. In order to reduce the number of experiments, an L_{16} (4^4) orthogonal design graph was used. In this way, only 16 experiments were necessary to run.

Centrifugation is a process where the gravity force is magnified to separate solids from liquids or one liquid from another[15]. Considering that α- amylase inhibitors were located in the cytoplasm of the cells, the applied centrifugal force led to tissue permeabilization by disrupting the important cellular structures such as cell walls and cell membranes, which are of great

importance for mass transfer control. Generally, an increase in the extraction of inhibitors was observed with an increase in the centrifugal force and this was due to the compaction of the solids as a result of higher centrifugal force[16]. The drop and rise of α-amylase inhibitory activity at 5000 and 6000 rpm may be due to some unknown synergetic factors. Hence, the optimal centrifugal force for the extraction of α-amylase inhibitors was found to be 4000 rpm. The main advantage of this type of extraction is its increased efficiency, which leads to increased yields and/or shorter extraction times. A positive correlation (r^2 = 0.790) was observed between centrifugal force and extraction time.

The extraction rate of α-amylase inhibitors was observed to decrease with an increase in time. This was may be due to the degradation of α-amylase inhibitors with an increased time and centrifugal force. The decrease in inhibition may be due to fact that α-amylase inhibitor compounds settles as debris[5]. Increase in time also led to an increase in the adhesion of particles around the walls of the supporting material like glass or plastic tubes during centrifugation[17].

The phenomenon of this type of extraction was governed by dissolution or diffusion kinetics. This kinetics is governed by driving force related to the gradient of component concentration between solid and liquid phase[18]. However, if the ratio of solvent to raw material reached a certain level, the extract may be well saturated with the solutes in the solution that may lead the extract to reach a steady state and may not increase significantly furthermore[19].

It was found that the percentage of α-amylase inhibition increases under slightly alkaline condition of pH 7.5 because of the presence of optimal concentration of α-amylase inhibitors. The inhibitory activity decrease from pH 6.0 to 6.5 and then a progressive increase in the activity was due to some synergetic factors. Generally, plant cell wall consists of xyloglucan polymers that are strongly hydrogen-bonded with cellulose fibrils, forming the only noncovalent link in the network of polymers which cross-link the cellulose fibers[20]. Naturally pH fluctuations stimulate cell wall weakening *in vitro* as well as *in vivo*. The ionization of a sugar hydroxyl group is negligible below pH 12, and since the protonation of the oxygen atom of a sugar hydroxyl is negligible above pH 1.0, hydrogen ions [H^+] would not be added to or subtracted from xyloglucan or cellulose. Moreover, analysis has revealed that a pH range from 2.0 to 7.0 may not alter the amount of xyloglucan fragments that has bound to cellulose. If the pH is increased beyond 7.0 the fragments of xyloglucan slowly reduced its tendency to interact with the cellulose fibrils, start to detach and move along the fibrils. This kind of movement was termed as "xyloglucan creep" that caused the destabilization of cell wall and leads the leakage of the components present in the cytoplasm[21].

In conclusion, the optimal process for the extraction of α-amylase inhibitors were found to be a centrifugal force of 4000 rpm, 5 min of centrifugation time, 1:20 solid-liquid ratio and pH 7.5. The maximum pancreatic α-amylase inhibitory activity has been recorded as 95.8% under the above mentioned optimal conditions. Moreover, centrifugal force was found to be a significant factor that affects the extraction procedure. The TLC results of the optimized extracts have revealed the presence of rutin and quercetin related flavonoids that may act as significant pancreatic α-amylase inhibitors. More research on the purification and structure elucidation of α- amylase inhibitors in the kernel of *Pouteria sapota* may be focused and carried out in the future.

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