Immobilization of trypsin on Chitosan Gels using different activation protocols and Comparison with other Supports.

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Abstract

In this work, trypsin was immobilized on chitosan gels prepared under different conditions: coagulation with 0.1M or 1M NaOH and activation with glutaraldehyde or glycydol. The obtained derivatives were characterized by their recovered activity, thermal (40, 55 and 70°C) and alkaline (pH 11) stabilities and amount of enzyme immobilized on gels (20-70mgE/gGel) and then compared with the soluble trypsin and with trypsin-agarose derivative. Immobilization yield and recovered activity of 100% were reached when trypsin was immobilized on gels activated with glutaraldehyde. Assays of thermal inactivation performed at 70ºC showed that the trypsin-agarose-glyoxyl derivative (TAGly, the reference derivative) was more stable than those prepared on chitosan and activated with glutaraldehyde and that the trypsin-chitosan-glyoxyl derivative (TQGly) was approximately three-fold more stable than TAGly one. Alkaline stability (pH 11.00) confirmed that TQGly was also the most stable derivative in this condition. Acid hydrolysis assays allowed observing that for the derivatives activated with glutaraldehyde, the enzyme molecules interacted with the support through several amino acids and that for the TAGly and TQGly derivatives, that contain glyoxyl groups, only a small number of enzyme-support bonds, via lysine amino acid, were formed. For all trypsin-chitosan-glutaraldehyde derivatives 100% of immobilization yield and recovered activity were observed up to 30mgE/gGel.

Introduction

The developing of methods to promote structural and functional stabilization of enzymes through immobilization is very important in order to increase the field of application of enzymes in industrial processes. There are a lot of methods to immobilize enzymes, but the immobilization/stabilization of enzymes by multipoint covalent attachment to pre-existing supports has presented much more practical advantages when compared with other immobilization methods, for example: the immobilization process may be easily controlled, different supports can be selected, the resulting derivatives should present less diffusion problems, among others. As consequence, these more rigid enzyme molecules are more resistant to conformational changes induced by heat and organic solvents than the corresponding unmodified ones.

Trypsin is an important proteolytic enzyme for the detergent and dairy industries (Galvão *et al.*, 2001). This enzyme was already immobilized on silica, porous glass, agarose and Celite™ (Bharadwaj *et al.*, 1992; Yanishpol'skii *et al.*, 1989; Guisán & Blanco, 1987; Huang *et al.*, 1997), but there are few reports about its immobilization on chitosan in the literature (Liu *et al.*, 2005).

Chitosan is a linear polysaccharide of high molecular mass composed of repeating units of β-(1-4)-2-amino-2-deoxi-D-glucopyranose (D-glucosamine). It is obtained via deacetylation of chitin (extracted from crab and prawn shell) with concentrated alkali, being usually referred as the

deacetylated derivative of the natural polymer chitin. Chitosan is an important by-product of the fishing industry and it presents great advantages in relation to other materials, mainly due to its great versatility, relative low cost and broad availability (Galvão, 2004).

In this work were developed some methods of immobilization of trypsin on chitosan gels using different methods of coagulation and activation of the support, aiming to the stabilization of the enzyme. The produced trypsin-chitosan derivatives were characterized and compared with other trypsin-agarose derivatives, following protocols available in the literature.

Material and Methods

Material

Powder of chitosan was purchased from Polymar Ind. Com. Exp. Ltda (Ceará-Brazil). Sepharose 6B-CL was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Trypsin (EC 3.4.21.4), from bovine pancreas, was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Benzoyl-L-arginine-p-nitroanilide was purchased from Bachem AG (Budendorf, Switzerland). Glutaraldehyde and Sodium borohydride were purchased from Quimibrás Indústrias Químicas S.A. (Brazil) and Nuclear (Brazil), respectively. All other reagents were of analytical grade.

Methods

Preparation of Chitosan Gel

Solution of chitosan 2.5% (mass basis) was prepared dissolving chitosan powder into acetic acid 5% (mass basis). This solution was filtered to remove impurities (small particles remainders of the chitosan obtaining process, from chitin) and, after that, the filtrate was dispersed through a nozzle into a coagulation solution (0.1 or 1M NaOH). The system was kept under stirring until complete coagulation of the particles. The resulting gel was washed with an excess distilled water using a stainless steel sieve (opening of 0.177mm) until pH 7 was reached. This preparation methodology followed protocol described by Arruda (1999).

Activation of Chitosan Gel

a) Chitosan gel coagulated with 1M NaOH was activated under two different conditions: i) glutaraldehyde 5% (mass basis) at 25°C and pH 7.00 (50mM sodium phosphate buffer) for 1 hour and ii) glutaraldehyde 5% (mass basis) at 25°C and pH 10.00 (100mM sodium bicarbonate buffer) for 1 hour, both under gently stirring.

b) Chitosan gel coagulated with 0.1M NaOH was also activated under two different conditions: i) glutaraldehyde 5% (mass basis) at 25°C and pH 7.00 (50mM sodium phosphate buffer) for 1 hour and ii) glycydol followed by oxidation with $NaIO_4$, according to preparation protocol of glyoxyl-agarose gel with low concentration of aldehyde groups (also referred to as low activation).

Preparation of Glyoxyl-Agarose and Agarose-Amine-Glutaraldehyde Gels

Glyoxyl-agarose gel poorly activated was prepared according to Guisán, 1988. Agarose-amine-glutaraldehyde gel was prepared according to Fernández-Lafuente et al., 1993.

Immobilization of Trypsin on Agarose and Chitosan Gels

Chitosan gels, activated as previously described, was added to an enzymatic solution prepared in 100mM sodium bicarbonate buffer pH 10.05 or 50mM sodium phosphate buffer pH 7.00, containing 3mM of benzamidine, in the ratio of V_{gel}/V_{total} of 1/10. This suspension was gently stirred on a shaker at room temperature (25ºC) for 24 hours. During the reaction time, aliquots of supernatant were withdrawn and their activity was measured. At the end of the period, sodium borohydride was added (1mg $NaBH_4$/mL of solution) to quench the reaction. After 30 minutes under mechanical stirring at room temperature, the produced derivative was washed with an excess

of distilled water and stored at 4ºC. Derivatives of trypsin on glyoxyl-agarose gel with low activation and agarose-amine-glutaraldehyde gel were prepared using a similar immobilization protocol. However, in the first case was offered a high enzymatic load (180mgE/gGel) and the enzyme/support contact time was of 20 minutes. In the second one, the contact time enzyme/support was of 2 hours. The obtained derivatives were firstly washed with 200mM sodium phosphate buffer, pH 7.00, and then with an excess of distilled water. The derivatives were stored at 4ºC.

Description of the Trypsin Derivatives

The prepared trypsin derivatives were nominated according to used support, as described below:

Denomination	Support	Coagulation solution	Activation Agent	Activation pH	Immobilization pH
TQGlu1M-A7	Chitosan	NAOH 1M	Glutaraldehyde	7.00	10.05
TQGlu1M-A10	Chitosan	NAOH 1M	Glutaraldehyde	10.00	10.05
TQGlu0.1M-Multip	Chitosan	NAOH 0.1M	Glutaraldehyde	7.00	10.05
TQGlu0.1M-Unip	Chitosan	NAOH 0.1M	Glutaraldehyde	7.00	7.00
TQGly	Chitosan	NAOH 0.1M	Glycydol and treated with $NaIO_4$	--	10.05
TAGly [(*)]	Agarose	--	Glycydol and treated with $NaIO_4$	--	10.05
TAaGlu	Agarose-amine	--	Glutaraldehyde	--	10.05

(*) Derivative prepared on glyoxyl-agarose gel with low concentration of aldehyde groups (Reference derivative unipontual).

Enzymatic Activity Assays

Enzymatic activity of soluble or immobilized trypsin was measured spectrophotometrically by monitoring the increase of the absorbance, at $\lambda=405nm$, during the hydrolysis of the synthetic substrate N-Benzoyl-L-arginine-p-nitroanilide (BApNA). Assays were performed in a 1-cm light path glass cuvette thermostatically controlled at 25°C, in a Ultrospec 2000 spectrophotometer (Pharmacia Biotech), adapted with magnetic stirring. 150μL of solution (soluble enzyme) or suspension (immobilized enzyme) of trypsin were added to 3mL of the synthetic substrate solution (0.4mM in sodium phosphate buffer 50mM and pH 8.00, in the presence of ethanol). The enzymatic activity was determined in Abs/min.

Irreversible Thermal and Alkaline Inactivation of Trypsin

Thermal inactivation: soluble enzyme and suspensions of the derivatives prepared in 50mM sodium phosphate buffer pH 8.00 were kept at different temperatures (40, 55 and 70ºC). Aliquots were withdrawn periodically and their enzymatic activity was measured as described previously until half-life time ($\tau_{1/2}$) was determined, at 25°C and pH 8.

Alkaline inactivation: soluble enzyme and suspensions of the derivatives prepared in 200mM borax-NaOH buffer, pH 11, were kept at 25ºC. Aliquots were withdrawn periodically and enzymatic activity was measured until half-life time ($\tau_{1/2}$) was determined, at 25°C and pH 8.

Investigation of the Capacity of Immobilization of Trypsin on Chitosan Gels

The capacity of immobilization of trypsin on chitosan-glutaraldehyde and agarose-amine-glutaraldehyde supports was investigated offering increasing initial enzymatic loads (20, 30, 40, 50, 60 and 70mgE/gGel). The activity in the supernatant was monitored during all the reaction time and the recovered activity was measured at the end of the process.

Acid Hydrolysis of Soluble and Immobilized Trypsin
Acid hydrolysis of soluble and immobilized trypsin was performed according to methodology described by Galvão, 2004.

Results and Discussion

Determination of the Yield of Immobilization and Recovered Activity

In this work, the yield of immobilization was defined as the ratio between the disappeared activity from supernatant during the immobilization process and the initial activity of the enzymatic preparation. The recovered activity was defined as the ratio between the measured activity on gel after immobilization and the disappeared activity from supernatant during the immobilization process.

According to Table 1, it can be observed that only two derivatives (TQGly and TAGly) didn't reach immobilization yield of 100% and complete recovery of the enzyme on support. A low immobilization yield was already expected to the TAGly derivative, because it was prepared on support with low concentration of aldehyde groups in its surface. However, in the case of TQGly derivative, it is possible that an excessive crosslink has occurred between the remaining amino groups in the support and the aldehyde groups generated on support after its activation with glycydol, reducing the internal area accessible to the protein.

Table 1: Immobilization yield and recovered activity on gels after 24 hours of interaction enzyme-supports.

| Derivatives	Initial activity (Abs/min)	Residual activity on supernatant (Abs/min)	Recovered activity (Abs/min)	Yield of immobilization (%)
TQGlu1M-A7 (10mgE/gGel)	0.0442	0	0.0445	≈100
TQGlu1M-A10 (10 mgE/gGel)	0.0442	0	0.0443	≈100
TQGlu0.1M-Multip (10 mgE/gGel)	0.0162	0	0.0170	≈100
TQGlu0.1M-Unip (10 mgE/gGel)	0.0162	0	0.0166	≈100
TQGly	0.1290	0.0510	0.0782	60.6
TAGly	1.385	1.1650	0.2220	16.0
TAaGlu	0.0150	0	0.0155	≈100

Irreversible Thermal and Alkaline Inactivation

The thermal inactivation was firstly performed in relatively low temperatures, 40 and 55ºC, where it was possible to follow with precision the loss of activity of the free enzyme and of the derivatives. For temperature of 70ºC only the derivatives were assayed because the soluble enzyme inactivates very quickly at this temperature. On the other hand, it was possible to follow the loss of activity of the derivatives and to differentiate them due to their higher thermal stability. Exponential models of first or second order were fitted to data of thermal inactivation for free and immobilized enzyme. Figure 1 shows the behavior of the following derivatives: TQGlu1M-A7, TQGlu1M-A10 and TAGly at 70ºC.
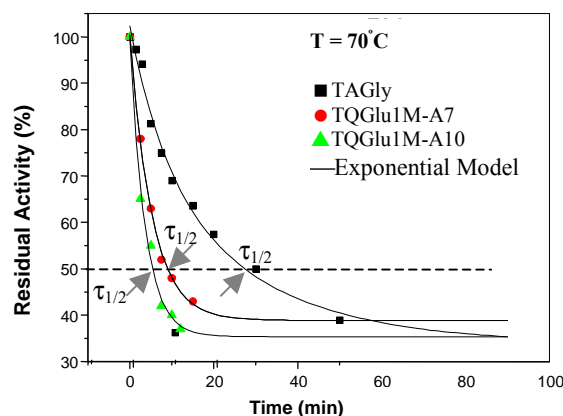
Figure 1: Residual activity at 70°C of the following trypsin derivatives: TQGlu1M-A10, TQGlu1M-A7 and TAGly.

The TAGly derivative was the most stable derivative assayed at this temperature, suggesting that the structure of the derivatives prepared on chitosan suffered a less intense rigidification after the immobilization process than that observed for the TAGly. Comparing the derivatives prepared on chitosan, the TQGlu1M-A7, whose support was activated with glutaraldehyde at pH 7, was more stable than the TQGlu1M-A10 derivative, whose activation with glutaraldehyde occurred at pH 10. Data from literature suggest that in alkaline pH (pH 9) there is a strong polymerization of glutaraldehyde on the gel surface. The reaction of the glutaraldehyde with the amino groups presents in the support may reduces its specific area (Monsan, 1978), decreasing the possibility of multi-interaction enzyme-support. Figure 2 shows the behavior of the derivatives TAGly, TAaGlu, TQGlu0.1M-Unip and TQGlu0.1M-Multip at 70ºC.
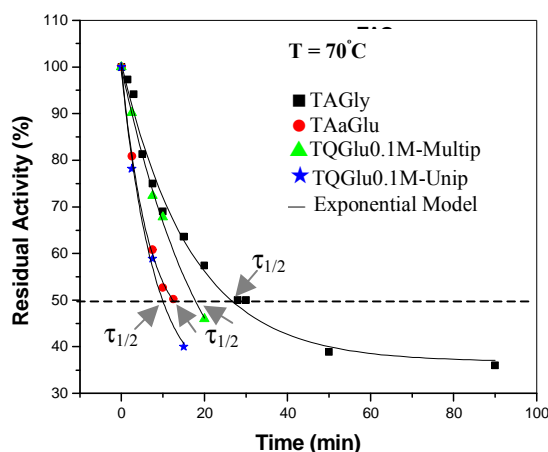

Figure 2: Residual activity at 70°C of the following trypsin derivatives: TQGlu0.1M-Unip, TQGlu0.1M-Multip, TAaGlu and TAGly.

It was expected a significant difference between TQGlu0.1M-Multip (immobilization at pH 10.05) and TQGlu0.1M-Unip (immobilization at pH 7) because the first one was immobilized at alkaline pH (10.05), a favorable condition for the establishment of enzyme-support multipoint linkages. However, at 70ºC, the TQGlu0.1M-Multip derivative was only 1.8-fold more stable than the TQGlu0.1M-Unip, showing that part of its structure became just a little bit more rigid by immobilization at pH 10.05. At 55ºC, both derivatives presented half-life time of 540 minutes, what shows that the difference between them is not very pronounced.
Figure 3 shows the decay of enzymatic activity for TQGly and TAGly at 70ºC. As can be observed, the TQGly derivative presented a half-life time of 85 minutes, approximately three-fold more stable

than the TAGly one, whose half-life time was of 28 minutes. The superiority of the TQGly derivative may be related to the methodology used for its preparation. The coagulation of the chitosan solution with 0.1M NaOH occurs more slowly, resulting in smaller particles and with more homogeneous porous, what increases the support superficial area available for the enzyme immobilization (Arruda, 1999).
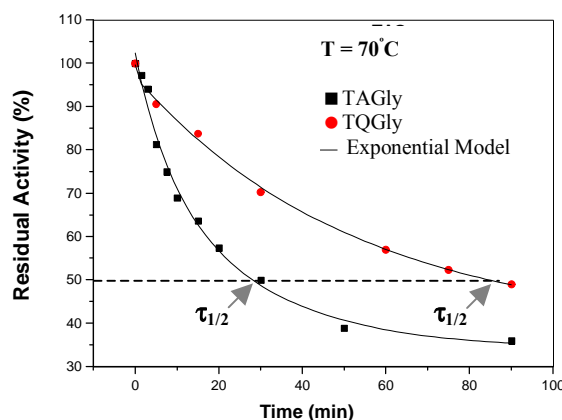
Figure 3: Residual activity for the TAGly and TQGly derivatives at 70°C.

Table 2 summarizes all the results obtained in the irreversible thermal inactivation assays:

Table 2: Half-life times ($\tau_{1/2}$) at 40, 55 and 70ºC and stability factors obtained at low temperatures (40 and 55ºC).

Specie	$\tau_{1/2}$ (min) at 40ºC	Stability Factors	$\tau_{1/2}$ (min) at 55ºC	Stability Factors	$\tau_{1/2}$ (min) at 70°C
Free Enzyme	20	1	4	1	-
TAaGlu	-	-	210	52.5	10
TQGlu0.1M-Unip	-	-	540	135	10
TQGlu0.1M-Multip	-	-	540	135	18
TAGly	-	-	1080	270	28
TQGlu1M-A10	720	36	-	-	6
TQGlu1M-A7	930	46.5	-	-	9
TQGly	-	-	2640	660	85

It is well known from literature that immobilization of enzymes on glyoxyl-agarose gels, with high or low concentration of aldehyde groups, results in derivatives with high thermal stability when compared with the soluble enzyme and with other preparation protocols. In this work, the TAGly derivative, prepared on glyoxyl-agarose gel with low concentration of aldehyde groups (unipontual), was used as comparison parameter to evaluate the stability of the trypsin-chitosan derivatives. At 55 and 70ºC all derivatives prepared on supports activated with glutaraldehyde were less stable than the TAGly derivative. However, the derivative prepared on chitosan gel activated according to protocol used to produce trypsin-glyoxyl-agarose derivatives was more stable than the reference derivative, TAGly, as can be seen in Table 2. All derivatives prepared on chitosan activated with glutaraldehyde (or ethylenediamine) presented low stabilization, including that one prepared on agarose-amine gel (TAaGlu). These results demonstrate the superiority of the method of activation with glycydol for production of supports for enzyme immobilization, although glutaraldehyde is still largely used (Park *et al.,* 2001; Jayakrishnan & Jameela, 1996).

Alkaline inactivation assays performed at 25ºC and pH 11, whose results are shown in Table 3, confirmed the tendencies observed in the thermal inactivation assays. Trypsin derivatives on chitosan-glutaraldehyde and agarose-amine-glutaraldehyde were less stable than the TAGly derivative (reference) and the TQGly derivative was again the most stable derivative, presenting half-life time 54 times higher than the soluble enzyme in the assayed conditions.

Table 3: Half-life times ($\tau_{1/2}$) and stability factors for soluble and immobilized trypsin at 25ºC and pH 11.

Specie	$\tau_{1/2}$ (hours) at pH 11	Stability Factor at pH 11
Free Enzyme	8	1
TAaGlu	55	6.8
TQGlu0.1M-Multip	107	13
TAGly	150	18.75
TQGlu1M-A10	48	6
TQGlu1M-A7	72	9
TQGly	437	54

Acid Hydrolysis of Soluble and Immobilized Trypsin

Acid hydrolysis of soluble and immobilized trypsin was performed aiming to investigate which kind of interaction occurred between the enzyme and the support. The obtained hydrolysates were analyzed by high performance liquid chromatography (HPLC) to quantify the released amino acids. As the acid hydrolysis cannot cleavages the covalent bonds established between enzyme and support, it was expected that the concentration of the lysine amino acid present in the derivatives' hydrolysates was smaller than that detected for the free enzyme's hydrolysate, indicating that this amino acid was, in fact, involved in the immobilization process. The obtained results are shown in Table 4.

Table 4: Concentration of some amino acids (μmol/mL) released after acid hydrolysis (HCl 6.8N, 105ºC and 24 hours) of soluble trypsin and different derivatives of this enzyme.

Amino Acid	Soluble Trypsin	Trypsin Derivatives Prepared on Different Supports				
		TAGly	TQGly	TAaGlu	TQGlu0.1M-Multip	TQGlu0.1M-Unip
Aspartic Acid	0.40	0.39	0.40	0.27	0.21	0.21
Threonine/ Asparagine	0.19	0.18	0.18	0.13	0.12	0.11
Serine	0.31	0.31	0.30	0.21	0.17	0.17
Lysine	0.12	0.09	0.10	0.02	0.06	0.05

The TAGly derivative, as suggested before, should be attached to the support by few bonds. This hypothesis was confirmed through the determination of lysine content in the hydrolysate of this derivative, which was only slightly smaller than the obtained for the soluble enzyme. Similar results were observed for the TQGly derivative, suggesting that this one was also immobilized on support by few bonds, as shown in Table 4.

The glutaraldehyde is a bifunctional agent that can react with several groups: hydroxyl, carbonyl, amine, sulphydryl and so on. For this reason, it is possible that the high reactivity of glutaraldehyde has allowed the immobilization of the enzyme by means of other amino acids, besides lysine, for the TAaGlu, TQGlu0.1M-Multip and TQGlu0.1M-Unip derivatives. This approach could explain the reduction in the content of all amino acid presented in Table 4.

Investigation of the Capacity of Immobilization of Trypsin on Chitosan Gels

The capacity of immobilization of trypsin on chitosan gels was investigated offering initial enzymatic loads from 20 to 70mgE/gGel to the following supports: TQGlu1M-A7, TQGlu1M-A10, TQGlu0.1M-Multip and TAaGlu. When enzymatic loads of 20 and 30mgE/gGel were offered, all initial activity was recovered on the gels (results not shown). Table 5 shows the results obtained for the remaining enzymatic loads. The immobilization time for these assays was of 24 hours.

Table 5: Initial and recovered activity for the different trypsin derivatives produced.

Enzymatic load (mgE/gGel)	Initial activity (Abs/min)				Recovered activity (Abs/min) [Percentage of recovering (%)]			
	40	50	60	70	40	50	60	70
TQGlu1M-A7	0.0386	0.0538	0.0795	0.0998	0.0390 [≈100%]	0.0388 [≈72.12%]	0.0556 [≈69.94%]	0.0598 [≈59.92%]
TQGlu1M-A10	0.0271	0.0375	0.0631	-	0.0225 [≈83.02%]	0.0305 [≈81.33%]	0.0431 [≈68.30%]	-
TQGlu0.1M-Multip	0.0695	0.0800	0.0947	0.1115	0.0692 [≈100%]	0.0766 [≈95.75%]	0.0694 [≈73.28%]	0.0815 [≈73.09%]
TAaGlu	0.0351	0.0582	0.0944	0.1282	0.0165 [≈47.01%]	0.0250 [≈42.96%]	0.0390 [≈41.31%]	0.0519 [≈40.48%]

According to the results presented in Table 5, 40mgE/gGel was the enzymatic load that provided the complete recovery of the initial activity for the derivatives TQGlu1M-A7 and TQGlu0.1M-Multip. For TQGlu1M-A10 and TAaGlu, it was recovered only 83.02% and 47.01% of the initial activity, respectively. It can also be observed that by increasing the enzymatic load, the percentage of recovery of the enzyme on the gels decreases. These results suggest that the limit of saturation of the gels may be close or has already been reached.

Conclusions

The immobilization of trypsin on gels activated with glutaraldehyde was efficient because it allowed recovery of 100% of enzymatic activity on the supports after immobilization and 100% of immobilization yield. Assays of irreversible thermal inactivation at 70ºC showed that the TAGly derivative (reference derivative), was more stable that the derivatives prepared on chitosan and activated with glutaraldehyde. But, at the same temperature, the TQGly derivative was approximately three-fold more stable than the TAGly. Irreversible alkaline inactivation (pH 11) confirmed that TQGly was also the most stable derivative in this condition. Acid hydrolysis assays showed that the TAGly and TQGly derivatives established a small number of bonds with the support, via lysine amino acid, and that for TAaGlu, TQGlu0.1M-Multip and TQGlu0.1M-Unip the enzymes molecules interacted with the support through several amino acids. Up to 30mgE/gGel all initial activity was recovered on all gels. For 40mgE/gGel, only the TQGlu1M-A7 and TQGlu0.1M-Multip derivatives allowed complete recovery of the enzyme on gel. Above this enzymatic load it was not possible to recover completely the offered amount of enzyme. While the results for the TQGly derivative are promising, more studies are needed to optimize its methodology of preparation, to characterize the support and to measure its performance as a biocatalyst.

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