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Plant-based coagulants for halal cheese production

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A B S T R A C T

Advancements in cheese-making technology have successfully integrated traditional methods with contemporary innovations, enhancing efficiency, sustainability, and product quality. Traditional cheese production typically uses animal rennet, which is sourced from the stomachs of young ruminants. This rennet contains the enzymes chymosin and pepsin, which are essential for the milk coagulation process in cheese making. Due to the limited availability of animal rennet and the rising demand for cheese and halal food products, the market has expanded beyond Muslim consumers to include non-Muslim individuals, leading to the exploration of alternative plant-based coagulants. Therefore, this study aims to evaluate the potential use of plant-based coagulants in producing halal cheese while identifying the challenges associated with the production process. These challenges include proteolytic activity, which can lead to a bitter taste, and inconsistencies in coagulant quality due to variations in the sources of the plants. The current study focuses on different types of plant proteases, such as aspartate, cysteine, and serine, extracted from different parts, as well as protease production techniques. It also explores coagulant quality parameters, such as milk clotting activity, proteolytic activity, optimal temperature, and pH, as well as their effects on the physicochemical and organoleptic properties of cheese. The results are expected to provide comprehensive scientific insights for the development of effective alternative coagulants to meet the needs of the halal cheese market in the future.

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1 Introduction

Over the years, cheese-making technology has advanced significantly, combining novel developments with traditional techniques to increase productivity, sustainability, and product quality. Initially, cheese production relied on coagulating agents, such as rennet, to convert fluid milk into a semi-solid mass. Rennet is traditionally produced by extracting young ruminant animals' stomach lining (abomasum), such as calves and lambs. In addition, it contains a mixture of 80% chymosin and 20% pepsin, depending on various factors, including animal's age at slaughter and diet (Nicosia *et al.* 2022). Chymosin is highly specific in hydrolyzing the peptide bond Phe105–Met106 within the caseinomacropeptide of kappa-casein (κ-CN), leading to the destabilization of casein micelles and subsequently inducing milk coagulation. Meanwhile, pepsin is rarely utilized due to its lower specificity in hydrolyzing peptide bonds, which can produce undesired residues (Jacob *et al.* 2011).

In 2023, global cheese production reached approximately 23 million metric tons, representing a consistent growth since 2015. The increasing global demand for cheese has led to challenges in meeting the need for rennet, primarily due to the limited availability of calf stomachs. In addition, the globalization of cheese markets has diversified consumer demands, requiring the industry to cater to various needs. These include the production of affordable products, including those with distinctive flavors and textures, and are also in line with halal standards (Alavi & Momen 2020).

In recent years, the demand for halal food products has extended beyond Muslim consumers to include non-Muslim individuals. This growing interest has driven the formation and expansion of the halal food sector, as halal products are widely perceived by non-Muslim consumers as safe, hygienic, high-quality, and wholesome (Nurrachmi 2017). However, ensuring the halal status of cheese products remains a significant challenge, mainly due to the difficulty in tracing the slaughtering process of the animals from which chymosin is sourced.

The development of chymosin from microbial sources has simplified the traceability of its halal status. However, microbial chymosin presents several drawbacks, including lower specificity, higher thermal stability, a reduced milk-clotting activity (MCA) to proteolytic activity (PA) ratio compared to calf rennet, and an increased likelihood of imparting bitterness (Nicosia *et al.* 2022). Addressing these limitations, chymosin production has advanced through genetically modified organisms (GMOs). Recombinant chymosin is functionally equivalent to animal-derived rennet and provides advantages in cost-efficiency and production consistency. Despite the potential, using GMOs in the production process raises ethical concerns, including consumer acceptability, labeling requirements, and long-term environmental and health impacts. These concerns have been extensively debated among regulatory agencies, industry stakeholders, and consumers (Lee 2015). As an alternative, plant-based milk coagulants are emerging as a viable solution to address the concerns.

Evaluating the effectiveness of plant-based milk coagulants requires an in-depth analysis of their performance compared to the specific properties of calf-rennet as a benchmark coagulating agent. Calf-rennet is characterized by its high MCA (specificity toward (κ-casein) relative to proteolytic activity, as well as thermolabile properties, ensuring that residual active coagulants are eliminated during whey processing (Jacob *et al.* 2011). Protease enzymes derived from various plants have been identified as potential milk coagulants, but their ability must meet the stringent criteria established by the specific properties of calf-rennet. Therefore, this study aims to identify the potential of plant-based coagulants in cheese production, evaluate the challenges in their application, and explore commercialization prospects.

2 Methodology

This study was carried out by conducting a comprehensive review of scientific literature to identify plant-based coagulants with potential applications in halal cheese production. Relevant sources, including peer-reviewed journal articles, books, and reports, were analyzed to evaluate the properties of key plant-derived protease enzymes, such as those from *Cynara cardunculus* (cardoon), *Moringa oleifera* seeds, fig latex, bromelain (pineapple extract), and melon extracts. The selected coagulants were then assessed based on their milk clotting activity (MCA), proteolytic activity (PA), optimal temperature, and pH conditions, as well as effects on cheese yield, texture, and organoleptic properties. Particular attention was given to challenges, such as bitterness and inconsistencies in enzyme quality, along with potential solutions to improve the applicability of these coagulants in halal cheese production.

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3 Types and Characteristics of Plant-Based Coagulants

Proteases were enzymes that could be extracted from various plant sources, as these played essential roles in multiple stages of a plant's life cycle. These enzymes could be used as plant-based milk coagulants in crude extract form or after purification (Kaur *et al.* 2024). Plant parts from which proteases could be extracted included fruits, seeds, latex, leaves, flowers, and rhizomes (Banik *et al.* 2018). Proteases were classified based on their catalytic mechanisms during hydrolysis, including aspartate, serine, cysteine, and metalloproteases. Notably, a study on plant-based milk coagulants had predominantly focused on the first 3 types of enzymes namely aspartate, serine, and cysteine proteases (Shah *et al.* 2014). Serine and cysteine proteases differed significantly in their catalytic mechanisms compared to aspartic and metalloproteases (Hofer *et al.* 2020). In serine and cysteine proteases, the nucleophile in the catalytic site was derived from an amino acid, while in aspartic and metalloproteases, it involved an activated water molecule (Bruno *et al.* 2006).

3.1 Aspartic Protease

Aspartic proteases were protease enzymes with catalytic mechanisms that played crucial roles in cheese production, including protein processing, maturation, and degradation (Yegin & Dekker 2013). These enzymes, which could be extracted from all living organisms, exhibited excellent milk-clotting and low proteolytic activity. Their catalytic properties closely resembled chymosin, including the specific cleavage of the Phe105–Met106 bond in κ-casein (Kumar & Sasmal 2020).

Milk coagulation, a critical step in cheese manufacturing, involved a 2-phase process facilitated by aspartic proteases. The enzyme specifically cleaves the Phe105–Met106 bond of κ-casein and splited the protein into 2 fragments namely para-κ-casein (hydrophobic) and casein glycomacropeptide (hydrophilic). The first phase was glycomacropeptide diffuses into the milk serum, causing the loss of its stabilizing effect. In the second phase, destabilized casein micelles aggregate in the presence of Ca^{2+} ions formed a gel, and this was susceptible to protein concentration, temperature, pH levels, and Ca^{2+} ion concentration, making the coagulation process susceptible to variations in the chemical environment (Yegin & Dekker 2013).

Aspartic proteases from different sources, including plants like *Cynara cardunculus*, *Cynara scolymus*, and *Helianthus annus*, had shown significant potential in cheese production and other biotechnological applications (Mazorra-Manzano *et al.* 2018; Ramadan *et al.* 2019). However, the catalytic characteristics of aspartic proteases varied depending on their source, emphasizing the need for further testing and evaluation to determine their efficacy as milk coagulants.

3.2 Cysteine Proteases

Cysteine proteases were characterized by the presence of a cysteine group in their active site, which played a key role in their catalytic mechanism. These enzymes exhibited activity across a broad range of pH levels and temperatures, making the enzymes promising candidates for applications in the food industry (Holyavka *et al.* 2021). However, their utilization came with certain drawbacks, including the need for reducing agents and chelating compounds, which could affect cost-efficiency (Markovic *et al.* 2023). Cysteine proteases were naturally abundant in various plant tissues, often occurring in significant quantities. Notable examples of plant-derived cysteine proteases that were successfully applied as coagulants in cheese production included papain, and ficin from *Ficus johannis* (Mohsin *et al.* 2024; Afsharnezhad *et al.* 2019). These enzymes had demonstrated potential as milk coagulants, though further evaluation was required to optimize their use in commercial cheese-making processes.

3.3 Serine Proteases

Serine proteases were characterized by a serine residue in their active site and exhibited some physiological characteristics such as symbiosis, protein degradation, and hypersensitivity response (Vidal *et al.* 2024). Plant-derived serine proteases were particularly suitable as milk coagulants due to their remarkable stability and activity under challenging conditions, such as elevated pH levels, high temperatures, and exposure to oxidizing agents or surfactants (Tripathi *et al.* 2011). These enzymes were distributed throughout various parts of plants, with the highest concentrations typically found in fruits. Examples of plant-based serine proteases included religiosin, dubiumin, streblin, cucumisin, and prunifoline, which were derived from diverse plant parts (Troncoso *et al.* 2022). Among these, dubiumin, extracted from Solanum dubium, had demonstrated effective milk-coagulating properties. Studies showed that dubiumin retained high stability across a wide pH range (4.0–11.0) and temperatures (20–90 ℃), making it a promising candidate for application in cheese production (Nicosia *et al.* 2022).

4 Production of Plant Proteases

The production of coagulants from plant sources was one of the alternatives in cheese making, which utilized protease enzymes. This process utilized protease enzymes extracted from various plant parts, such as flowers, seeds, roots, and leaves, to replace the limited and expensive animal rennet. With proper extraction and purification techniques, plant-based coagulants could provide effective milk coagulation for various types of cheese. The methods to produce coagulants for cheese included the following.

4.1 Production from Natural Sources

The production of coagulants from natural sources could be done by utilizing protease enzymes extracted from various plant parts such as flowers, seeds, roots, and leaves. The first stage involved collecting and preparing plant materials known to contain protease enzymes, such as Moringa leaf seeds, which were then dried and pulverized into powder. This was followed by an extraction process using supercritical carbon dioxide to purify the protein and remove fat. Protein extraction was then done by immersing the powder in NaCl solution and centrifuging. In addition, to achieve a higher level of purity, the fractions were processed using preparative HPLC. This stage produced protease with high activity and good stability (Wang *et al.* 2020). The protease in moringa seeds belongs to the aspartic protease type. Plant proteases played an essential part in cheese ripening at an early stage, concerning their primary activity in milk coagulation, anti-oxidant properties, and significant control over lipid oxidation (Sharma *et al.* 2023). Almost all enzymes used for milk clotting were aspartic proteases, such as milk-clotting proteases from *Cynara scolymus* leaves, *Carduus defloratus* flowers, sour orange *Citrus aurantium* L. flowers, and *Streblus aspler* twigs (Esposito *et al.* 2016).

4.2 In-vitro Production

In-vitro production utilized cell and callus cultures from various plants to produce protease enzymes for milk curdling. This culture was developed to overcome the availability of enzymes from natural sources influenced by the environment or the plants' season. A study by Alavi & Momen (2020) showed that plant coagulants could be extracted from *Cynara cardunculus*, which contained cardosin, which protease enzymes triggered high milk clotting activity. The production of protease from *Cynara cardunculus* flowers was carried out by preliminary extraction under acidic conditions to increase milk clotting activity and the ratio of clotting activity to proteolytic activity. Recombinant DNA technology enabled large-scale production of proteases through microorganism expression or plant cell culture, providing better quality control and product consistency. Plant tissue culture techniques allowed in vitro production of enzymes under controlled conditions without the need for fresh flowers. This could reduce the variability that often occured in natural raw materials. This approach improved process efficiency and ensured enzyme availability for producing high-quality cheese.

5 The Quality Analysis of Plant-Based Coagulant

The quality of plant-based coagulants used in cheese production depended on several parameters. These parameters ensured that the coagulant could produce the desired physicochemical properties and optimal results in the coagulation process. The following were some of the main parameters that determined the quality of plant-based coagulants for cheese.

5.1 Milk Clotting Activity (MCA)

MCA measured the ability of a coagulant enzyme, such as a plant-based peptidase, to induce clot formation in milk. This parameter determined how quickly and effectively a coagulant could trigger milk coagulation to produce curd (clumped milk solids). In the study of Kumar & Sasmal (2020), a high ratio of MCA/PA values indicated that pumpkin seed extract was effective for the gel formation of milk without causing excessive protein breakdown that could produce a bitter taste. A lower ratio resulted in less than optimal curd recovery and affected the final product yield (Nasr *et al.* 2016). Protease enzymes from *Cynara cardunculus* flowers in the study of Alavi & Momen (2020) had excellent milk clotting activity under certain conditions. Compared to chymosin, the coagulant from *Cynara cardunculus* had a higher MCA/PA ratio. In addition, the drying process of the flowers before extraction could decrease the MCA activity, and extraction from fresh flowers was preferred for maximum yield. A protease enzyme isolated from *Moringa oleifera* seeds showed optimal MCA at 60 ℃ and pH 5 (Wang *et al.* 2020). This process involved purification by HPLC, where the MCA of the enzyme increased significantly compared to that of the crude extract.

Citrus uranium Flower Extract (CAFE) showed MCA/ PA over a wide temperature range (35-70 ℃) in milk. The MCA/PA ratio was sufficient to cause milk coagulation like the commercial rennet (Khan *et al.* 2019). Fig latex contained ficin enzyme whose application, mechanism of action, and physical properties still needed to be explored. The study interest in ficin was increasing due to its proteolytic extract having active fragments that produce antibiotics, milk clotting, promiscuous activity, and meat tenderization. The plant extracts of figs had gained importance related to the health perception of consumers (Tahir *et al.* 2023). Bromelain had a higher MCA/PA ratio with an optimum pH range between 6 and 7

at 70℃ for milk coagulation. The milk coagulation activity of bromelain showed stability over a wide range of pH, therefore, it was effective over the entire gastrointestinal tract (Singh *et al.* 2023). This was safe and non-toxic, but exploring its potential in food products was necessary to gain health advantages (Imran & Alsayeqh 2022). The melon extract from the fresh sarcocarp portion showed a higher MCA/PA ratio at optimum pH and temperature, but MCA declined at lower temperature and pH levels (Khan *et al.* 2023).

5.2 Proteolytic Activity (PA)

Proteolytic activity measured the ability of enzymes to break down proteins, especially casein, in milk. In this coagulation process, proteolytic activity must be controlled because too much protein breakdown could produce a mushy cheese texture and bitter taste. The proteolytic activity of pumpkin seed extract was relatively balanced and produced strong milk gel without giving a bitter taste (Kumar & Sasmal 2020). High PA could increase the flavor intensity of cheese, but excessive protein digestion could cause bitterness. The bitter taste resulted from hydrophobic peptides from excessive protein breakdown. This appeared mainly in cheese made from cow's milk compared to goat's and sheep's milk, which were more resistant to bitter peptides (Alavi & Momen 2020). PA from *Cynara cardunculus* flower extract had lower activity than MCA, but the level of proteolysis was controllable enough to support optimal curd formation without producing bitterness (Alavi & Momen 2020). A protease from *Moringa oleifera* seeds had a fairly good and controllable PA, making it a milk coagulant in cheese making. Compared to aspartic protease from *Mucor bacilliformis* (Km = 3.88 mg/mL), protease from *Moringa oleifera* had a higher affinity for casein. However, its affinity was lower than metalloproteinase protease from *Paenibacillus spp.* (Km = 1.36 mg/mL) (Wang *et al.* 2020). The high affinity for casein and the ability to work at optimal temperature and pH made it efficient in the coagulation process and minimized the bitter taste due to excessive proteolysis..

5.3 Optimum Temperature and pH

A suitable coagulant must work in a specific pH and temperature range that matched the natural conditions of milk. The optimal temperature for rennet activity in the milk coagulation process ranged from 35-40℃ (Ben *et al.* 2017). Lower pH, such as pH 2, could reduce milk coagulation activity by about 60%. Acid coagulation occurred in the blank buffer of pH 2.0-3.0, although coagulation was not complete (Wang *et al.* 2020). Protease played an important role in coagulation because when adding protease, milk clotting occurred immediately. Temperature and pH were related to MCA, where pumpkin seed extract had optimal activity at pH 6.8 and temperature of 54℃, which was suitable for the milk curdling process (Kumar & Sasmal 2020). The pH and temperature made pumpkin seed extract more flexible for application under certain pH conditions.

The enzymes produced cheese with a smooth texture and more intense aroma at optimal pH. Coagulation did not occur appropriately when the pH was not maintained, and excessive proteolysis could damage cheese quality (Alavi & Momen 2020). MCA and PA were pH-sensitive, which showed how the milk environment affected protease enzyme properties. A study by Alavi & Momen (2020) showed that at temperatures above 65℃, enzymes began to denature and caused a significant decrease in activity. At low temperatures (around 25℃), milk coagulation with the coagulant from Cynara cardunculus proceeded faster than the coagulant chymosin. In terms of acidity, optimal activity was reached at pH 5, while the activity decreased gradually as the pH increased and disappeared entirely at pH 7. This suggested that this enzyme worked most effectively in a mildly acidic environment.

There was also a study by Wang *et al.* (2020) that explained that the protease enzyme obtained from *Moringa oleifera* showed optimal activity in the milk coagulation process at pH 5 and 60℃. Mild acidic conditions at pH 5.0 allowed the enzyme to work optimally in inducing curd formation, with a range between pH 4.0 to 7.0. The activity decreased above pH 7.0 because the enzyme became inactive in an alkaline environment. Regarding temperature, the enzyme activity was highest at 60℃, which was suitable for high-temperature milk processing, including pasteurization. The enzyme also showed good thermal stability, with more than 92% activity maintained after incubation at 50-80℃ for 1-2 hours. High thermal stability was an important factor for proteases for industrial processes (Wang *et al.* 2020). A study by Khan *et al.* (2024) showed that plant extract-based cheddar cheese samples showed similar pH trends and showed no significant difference with the control treatment made using the rennet enzyme. There was a decrease in pH from 6 to 5.52 during the cheese production process, which was necessary to continuously monitor the pH during curd formation, with drainage, and ripening for the proper maturation of cheese samples. These plant enzymes exhibited a drop in pH after 2 months of storage when cheese samples were added with additives to support texture firmness (Grossmann & McClements 2021). The higher time temperature treatments during the curdling process could lead to acidic pH, which could cause a problem in the rheological properties of cheese during maturation (Yano & Fu 2022). In particular, milk-clotting proteases could potentially be catalysts in the cheese industry. This property made it a potential alternative milk coagulant in cheese making, especially in sectors that required enzyme stability under diverse thermal and pH conditions.

6 Effect of Plant-Based Coagulants on Cheese-Making

Plant-based coagulants had long been used in cheese production, such as vegetable-based cheese from Spain and Portugal made with coagulants derived from *Cynara spp.* extracts (Ordiales *et al.* 2014). However, using crude extracts as coagulants posed several challenges in cheese production. Excessive proteolytic activity resulted in continuous hydrolysis of casein, particularly α- and β-casein, leading to lower cheese yields and undesirable bitter flavors and textures (Leulmi *et al.* 2023). Therefore, it was crucial to evaluate the components that influenced the quality of cheese produced using various types of plant-based coagulants, including yield, physicochemical components, organoleptic properties, and texture.

6.1 Yield

Cheese yield was influenced by various factors, including milk composition and quality, the type of cheese, and processing methods (Cipolat-Gotet *et al.* 2013). The use of plant-based coagulants was included as a factor within processing methods. In cheese production, proteases with a higher MCA/PA ratio resulted in curd with greater yield and reduced bitterness (Ariskanopitasari *et al.* 2023). Conversely, a lower ratio causes reduced curd hardness, poor curd recovery, and the release of bitter peptides that altered the final product's organoleptic qualities (Kumar & Sasmal 2020). Several studies had found that cheese made with plant-based coagulants yielded lower outputs than the one made with animal rennet (Shah *et al.* 2014). However, exceptions to this trend existed that plant-based coagulants from fig latex, *Cynara* extracts, and *Solanum elaeagnifolium* extracts had been shown to produce higher cheese yields compared to chymosin-based cheese (Mazorra-Manzano 2013).

6.2 Physicochemical Properties

The physicochemical properties of cheese included pH, fat content, protein content, lactose content, moisture, total solids, syneresis, salt content, and mineral composition (Muresan *et al.* 2021). These physicochemical properties determined the final quality of cheese products, including texture, flavor, and shelf life. Changes in physicochemical characteristics occurred during ripening, giving the cheese a soft texture and a mildly peppery flavor (Amaniyah *et al.* 2024). This highlighted how the hydrolytic activity of plant proteases on caseins significantly affected the characteristics of curd and cheese. However, prolonged exposure to proteases could result in the proteolytic breakdown of the casein network, particularly α- and β-casein, reducing curd yield by approximately 0.3% to 0.7% (Nicosia *et al.* 2022).

Several studies had reported that cheese made with crude plant extract coagulants exhibited physicochemical properties nearly identical to cheese made with commercial rennet. For instance, physicochemical properties such as pH, acidity, and fat content tested in Camembert cheese made with raw cardoon flower extract showed no significant differences from cheese made with commercial rennet (Zikiou & Zidoune 2019). Similarly, latex peptidases from *Calotropis procera* produced cheese with dry mass and soluble protein levels comparable to those made with chymosin (Freitas *et al.* 2016). Environmental factors, such as soil type and climate, significantly influenced the composition of plant-based coagulants, even within the same plant variety (Roseiro *et al.* 2003). These environmental factors, which were challenging to standardize, led to variations in the characteristics of cheese products. Therefore, optimizing the production of plant-based coagulants at the industrial level was essential to ensure consistent composition and quality (Benalia *et al.* 2024).

6.3 Organoleptic Properties

The organoleptic properties of cheese resulted from complex biochemical processes during cheese ripening, primarily involving proteolysis. Furthermore, this generated distinctive flavors, aromas, and textures in cheese. Variations in organoleptic characteristics were often linked to specific hydrolysis reactions of kappa-casein (κ-casein) and the rate of hydrolysis (Murlidhar *et al.* 2017). High proteolytic activity in plant-derived protease enzymes was the main cause of undesirable organoleptic properties, such as an overly acidic or bitter taste and excessively soft or hard textures (Aktayeva *et al.* 2018). Bitter flavors in cheese were caused by the formation of short peptides (<1 kDa) during hydrolysis under conditions of high proteolytic activity (Kuhfeld *et al.* 2023). Some cheese types, such as blue cheese, deliberately incorporated a distinctive and unique bitterness during extended fermentation, and this was generally considered an undesirable attribute in most cheese products (Mohsin *et al.* 2024).

A study by Delgado-Martinez *et al.* (2019), explored the use of preservation techniques such as High-Pressure Processing (HPP) as a solution to prevent excessive ripening in cheese caused by high proteolytic activity when using plant extracts as coagulants. HPP could reduce enzymatic and microbial activity during cheese ripening, slowing lipolytic and proteolytic reactions. This delayed casein degradation, which was

crucial for controlling texture formation and developing undesirable flavor compounds (Ribeiro *et al.* 2018).

6.4 Textural Properties

Texture was a primary factor consumers considered when selecting cheese products, as it was the most visually distinguishable characteristic among different types of cheese. This texture could be assessed using various methods, including textural profile analysis (TPA) with a texture analyzer. (Mohsin *et al.* 2024). This evaluation method measured texture based on hardness, cohesiveness, springiness, and chewiness. A study by Alavi & Momen (2020), assessed the texture of cheese made with plant-based coagulants compared to cheese made with commercial chymosin. The study revealed that cheese made using thistle flower coagulant had superior textural qualities, exhibiting increased softness and reduced hardness compared to cheese produced with commercial chymosin coagulants.

7 Conclusions

In conclusion, using plant-based coagulants for milk coagulation in cheese production offered an alternative solution for creating products with traceable halal status. However, their application presented challenges, including bitterness caused by excessive proteolytic activity and inconsistencies in coagulant quality due to the variability of plant sources. This was essential to implement cost-effective strategies to reduce defects in cheese produced with plant-based coagulants to enhance their commercial potential. This approach could ensure both quality and market sustainability.

Conflict of Interest

The authors declare no conflict of interest.

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