# EFFECTS OF ENZYMATIC CLARIFICATION OF POMEGRANATE JUICE BY PROTEASE AND PECTINASE TREATMENTS

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## ABSTRACT

This study analyses the efficiency of pectinase and protease enzymes used in the clarification treatments of pomegranate juice. The enzymatic treatment produced a significant reduction of turbidity. The combination of pectinase and protease gives higher reduction of turbidity. Among three concentrations of pectinase and protease (0.05, 0.25 and 0.5 % (v/v)), 0.5 % produced a juice with the higher clarity. Overall the quality of pectinase-treated pomegranate juice was better when compared with untreated or protease-treated juice. Our results suggest that the proteins play a decisive role in the formation of immediate turbidity in pomegranate juice, and point to that pectin may contribute to turbidity development during cold storage of pomegranate juice. Thus, the data may pave the way for development of improved alternative procedures for pomegranate juice clarification.

**Keywords:** Pomegranate juice; Clarification; Pectinase; Protease.

**No:of Tables: 1** **No:of References:20**

## Introduction

The pomegranate juice has potential health benefits such as anti-atherosclerotic and anti-inflammatory effects in mice that may be attributable to its anti-oxidative properties (Faria et al., 2007). The antioxidant capacity of commercial pomegranate juice is three times higher than those of red wine and green tea (Gil et al., 2000). Thus, this fruit juice is consumed in large quantities all over the world. One of the greatest hindrances to the marketability of this juice is its turbid appearance, which makes it undesirable to consumers.Fruit juices contain colloids that are mainly polysaccharides (pectin, cellulose, hemicelluloses, lignin and starch), protein, tannin and metals (Vaillant et al., 2001). One of major problems encountered in the preparation of fruit juices is the cloudiness due primarily to the presence of pectins. A fresh juice of pomegranate contained 14 mg of pectin in litre of juice (El Nemr et al., 2006). The pectin can be associated with plant polymers and the cell debris which are fiber-like molecular structure. The cloudiness that they cause is difficult to remove except by enzymatic depectinization. Several researchers have reported that depectinization using pectinase could effectively clarify fruit juices (Alvarez et al., 1998; Ceci et LOZANO, 1998; Vaillant et al., 1999; Yusof and Ibrahim, 1994). Pectinase hydrolyzes pectins and leads pectin–protein complexes to flocculate. The resulting juice has a much lower amount of pectins and a lower viscosity, which facilitates the subsequent filtration process.The purpose of the present study was to investigate the effect of commercially available pectinase and protease on turbidity and clarity of pomegranate juice. We particularly targeted an application using enzymes able to catalyze the degradation of protein and pectin structures.

## Materials And Methods

### Chemicals and reagents

Folin-Ciocalteu reagent, Copper (II) sulfate pentahydrate $CuSO_4 \cdot 5H_2O$ and sodium hydroxide were from R&M Chemicals (Essex. UK)., Gallic and tannic acids, 3- hydroxybiphenyl, bovine serum albumin, the pectinase from Aspergillus niger, with activity ≥1 units/mg and the Protease (from Aspergillus saitoi, with activity ≥ 0.6 units/mg were supplied by Sigma–Aldrich (St. Louis, MO, USA). These enzyme preparations were selected on the basis of results obtained previously with experimental black currant juice clarification (Landbo et al., 2006).

### Juice samples

Pomegranate juice samples were extracted from the cultivar Sefri planted in 1996 and provided by the INRA, Experimental Station, Meknes-Morocco. The fruits were peeled and the skins covering the seeds were removed manually. The juice was obtained from pomegranate arils by mechanical press. Three replicates were maintained for each analysis.

**Experimental clarification treatments**

The clarification treatments used 150 ml of pomegranate juice, stirred with different concentrations (0.05, 0.25 and 0.50% v/v) of pectinase and protease enzymes. Then, the enzyme-treated juices were incubated in a water bath at 50°C for 2 h. The enzyme-treated juices were then placed in a boiling water bath for 5 min to inactivate the enzyme. The enzyme-treated juices were then rapidly cooled by cold water to 25°C, then tested and analyzed immediately.

**Titrable acidity, pH and total soluble solids**

The titrable acidity (TA) was determined by titration to pH 8.1 with 0.1M NaOH solution and expressed as grams of citric acid per 100 ml of juice. The pH measurements were performed using a digital pH meter (Thermo Orion 3 star) at 21 °C. The total soluble solids (TSS) were determined with a digital refractrometer (Metteler-Toledo Gmbh, 30 PX, Switzerland) calibrated using distilled water). The results were reported as °Brix at 21 °C. The ratio TSS/ acid was also determined.

**Turbidity measurements**

The turbidity of the juice was determined using a portable Turbidimeter (MODEL 2100 AN - HACH Company) and results were reported in Nephelometric Turbidity Units (NTU).

**Clarity**

The clarity of the juice obtained was determined by measuring the absorbance at a wavelength of 660 nm using a Safas UV-Visible spectrophotometer. Distilled water was used as the reference.

**Determination of pectin content**

The total pectin in the juice samples was evaluated according to the method described by Filisetti-cozzi and Carpita (1991). Briefly, 0.2 ml of 125-fold diluted juice was mixed with 20 µl of 4 M sulfamic acid solubilized in KOH (pH 1.6) and 1 ml of 96 % sulfuric acid. The samples were then heated at 80 °C for 1h. Then, 200 µl of 3-hydroxybiphenyl reagent, diluted to 1:50 (w/v) with 80 % sulfuric acid, were added and the absorbance was measured at 525 nm over 5 min. The pectin concentration was expressed as d-galacturonic acid equivalents in mg/ml.

**Lowry procedure for protein content determination**

The total protein content was evaluated by the Lowry method (Lowry et al., 1951). The protein biuret reaction was carried out using reagent A containing 20 g of sodium hydroxide, 100 g of sodium carbonate and 2 g of Seignette salt and 0.5 g of copper (II) sulphate (pentahydrate) per 1000 ml in distilled water. 1 ml of prepared assay sample and 1 ml of reagent A were mixed and allowed to stand for 10 min at room temperature. Then four millilitres of diluted Folin reagent, prepared by dilution of 0.5ml of 1N Folin reagent with 4 ml of

distilled water, were added to the mixture. The solution was incubated for 5 min at 55 ± 1 °C and allowed to cool in bath of cold water for 30 min. The solution absorbance was measured at 670 nm. The total protein content of the juice was calculated from a calibration curve prepared with bovine serum albumin.

**Total phenol content determination**

The total phenolics (TP) were determined by using Folin–Ciocalteu method (Singleton et al.,1965). Briefly, 300 µl of diluted pomegranate juice in the ratio of 1:100 with methanol: water (6:4) was mixed with 1.5 ml of 10-fold-diluted Folin–Ciocalteu reagent and 1.2 ml of 7.5 % sodium carbonate. The mixture was incubated for 90 min at room temperature and the absorbance was measured by a Safas UV-Visible spectrophotometer at 760 nm. Gallic acid was used as a standard. The results were expressed as mg gallic acid equivalent in liter of fruit juice (mg GAE/L of juice).

**Anthocyanin content (TAC).**

The total anthocyanin content (TAC) was evaluated by applying by the method described by Ozgen *et al.,*(2008) using two buffer systems: sodium acetate buffer pH 4.5 (0.4 M) and potassium chloride buffer pH 1.0 (25 mM). Briefly, 0.4 mL of pomegranate juice was mixed with 3.6 mL of corresponding buffers and the absorbance was determinated at 510 and 700 nm. The water was used as a blank. The Absorbance (A) was expressed as:

$$A = (A_{520nm} - A_{700nm})pH_{1.0} - (A_{520nm} - A_{700nm})pH_{4.5}$$

TAC of juice was presented as mg cyanidin-3-glucoside $L^{-1}$ of PJ and determinated using equation below:

$$TAC = [A \times MW \times DF \times 100] \times 1/MA$$

with: A: absorbance; MW: molecular weight (449.2 g $moL^{-1}$); DF: dilution factor (10); MA: molar absorptivity of cyanidin-3-glucoside (26.900).

**Antioxidant activity by DPPH assay.**

The antioxidant activity of the pomegranate juice was achieved by method of Brand-Williams et al. (1995). Briefly, 100 µL of juice diluted at 1:100 with methanol:water (6:4), 2 mL of 0.1 mM DPPH in methanol were mixed. After 30 minutes of reaction, the absorbance was determined at 517 nm. For the background correction, the mixture prepared without DPPH. The antioxidant activity was determined according the equation below :

Antioxidant activity % = [1− (Abs sample 517 nm/Abs control 517 nm)] ×100

**Results and Discussion**

**Effect of the clarification treatments on pomegranate juice turbidity**

The individual turbidity-reducing effects of pectinase and protease treatments were

statistically significant immediately after the treatment at ($P<0.05$), Table 1 shows that the turbidity values were mainly dependent on enzyme concentration. An increase in enzyme concentration drastically decreased turbidity. The tubidity of juice sample treated by pectinase was lowest than those of the juice treated by protease for all concentration of enzymes (0.05, 0.25, 0,50 % v/v). While, the untreated sample had a higher turbidity than the clarified juice by enzymes. It has previously been reported that pectinase treatment may result in either increased or decreased turbidity of fruit (Landbo et al., 2006; Siebert, 2006). This phenomenon may be linked to the paradox that the pectinolytic enzyme attack can result in two different events (Siebert, 2006): The pectinases catalyze the degradation of high molecular weight pectin structures into small pectin fractions. The resulting small, negatively charged, pectin fractions may be able to stay in suspension and contribute to increase the immediate turbidity due to an increased scatter effect of small particles as compared to larger. The pectinases act on the pectin layer encapsulating the protein core of proteinaceous pectin particles in suspension. This action results in an electrostatic agglomeration of oppositely charged particles that may lead to transient turbidity increase and subsequently results in precipitation of the agglomerated complex resulting in decreased turbidity.The effect of the pectinase clarification treatment will then be a balance or rather a competition between the formation of small suspended particles, the agglomeration of oppositely charged pectin–protein structures, and the spontaneous precipitation of the latter structures. Based on the above theories, the influence of pectinase clarification treatment on immediate turbidity of juices thus seem to depend on the nature of the suspended pectin particles, the compositional and structural features of the particular pectin present in the juice, e.g. charge and degree of methylation, degree of polymerization, and the extent of association with protein. These pectin properties may vary as a result of pre-press pectinase treatments of juices and the pH of the juice. Nevertheless, the fact that the pectinase treatment decreased turbidity during cold storage of juices may be considered novel in relation to the pertaining theory that the main clarification action of pectinases is to decrease the immediate turbidity by promoting the agglomeration of oppositely charged pectin–protein particles (Grassin and Fauquembergue, 1996). Thus, as we have previously suggested for black currant juice, it is likely that protein–pectin interactions can also influence the development of turbidity during cold storage of juice (Landbo et al., 2006), even though the main cause for haze formation in juices may be a result of protein–polyphenol interactions (Siebert, 2006). The clarifying effect of the protease treatment on the immediate turbidity of the pomegranate juices was also a new result. The protease catalyzed hydrolysis of the pomegranate juice proteins was presumed to mainly prevent the formation of protein–

polyphenol complexes during cold storage. It was particularly intriguing that the protease addition to pomegranate juice consistently gave the higher clarity level compared to the untreated juice.The reduction of turbidity of juice after protease treatment may be the result of the protease catalyzed hydrolysis of pomegranate juice proteins having prevented the formation of complexes between positively charged proteins and negatively charged pectins. This complex formation would otherwise contribute to increase the immediate turbidity at least transiently. The insolubility and turbidity-causing effects of these complexes or of complexes resulting from e.g. the association of proteins with cell materials and/or phenolic compounds might be alleviated by the proteolytic action. The protease may also have acted by catalyzing the hydrolytic degradation of intra-cellular organelles and cell membranes of protein nature, such as e.g. the tonoplast surrounding the cell vacuoles or the extensins forming the cell wall.

**Effect of the clarification treatments on pomegranate juice clarity**

The effect of different enzyme treatment on the clarity was reported in the table 1. The values were significantly affected by pectinase and protease concentration at ($p< 0.05$). The pectinase and protease treated juices were free from sediments and consistently clear as indicated by low values of absorbance at 660 nm for the juice treated by pectinase that ranged between 0.048 and 0.021 and for protease ranged among 0.053 and 0.029, while it's greater for the untreated juice. However, the clarity of pomegranate juice was found to increase with an increase in enzyme concentration. The mixture of the two enzymes showed a greater increase in juice clarity compared to juice treated by a single enzyme. In general, the time required to obtain a clear juice is inversely proportional to the concentration of enzyme used at constant temperature (Kilara, 1982).

**Titrable acidity, pH and total soluble solids**

The following discussion of the chemical characteristics for untreated and enzyme-treated pomegranate juice is based on the data given in Table 1. The pH of pomegranate juice treated with the pectinase ranged from 3.69 to 3.83 and with protease ranged from 3.72 to 3.87 showing a decrease in pH values. The decrease in pH was directly related to increasing pectinase and protease concentration. TSS (g/ kg) of pectinase- and protease-treated juice were greater than the untreated juice. The increase of TSS was obvious with increasing of pectinase and protease enzyme concentration. This increase of TSS was attributed to the greater degree of tissue breakdown, releasing more components that contribute to soluble solids (Tung-Sun, et al., 1995). The TSS/acid ratio is the major analytical measurement for quality in pomegranate juice. The TSS/acid ratio of pomegranate juice was increased by increasing of pectinase and protease concentration. TSS/ acid ratio was shown

to be correlated with sweetness but not so closely with flavour (Guyer et al., 1993). Titrable acidity of pectinase- and protease-treated juice was higher than of untreated juice (Table 1), which may due to enzymatic deesterification and degradation of pectin resulting in an increase of total acid.

**Total phenols, pectin, and protein contents**

The analyses of the total phenols, pectin levels, and protein levels showed that these analytical data reveal any consistent drop or increase in the measured levels of the differently treated pomegranate juice samples (Table 1). The finding that the measured levels of total phenols, total pectin, and total protein were similar in the untreated and in the experimentally treated samples (Table 1), indicated that the influence of the different clarification treatments most likely reflected differences in the size and perhaps the shape of the turbidity causing molecules and not in their total content. Certainly, enzyme catalyzed pectin and protein hydrolysis may in fact not lead to a drop in pectin or protein levels, but rather just change their reactivity via change of charges and sizes via depolymerization and in the case of pectin also via demethylation trop longues phrases. Naturally occurring fruit polyphenols, including those present in fruit juices and pomegranate fruit extracts, exert antioxidant activity (Gil et al., 2000). This antioxidant activity has been linked to the prevention of the initial steps of certain pathologies related to coronary heart diseases, ageing and certain cancers (Benzie, 2000). It is therefore desirable to keep the phenolic levels as high as possible in commercial fruit juices.

**Anthocyanin content and Antioxidant activity**

The TAC are water-soluble pigments primarily responsible for the attractive red-purple colour of many fruits, including pomegranate juice, and they are well known for their antioxidant capacity. As shown in the table 1, there was a low decrease in terms of anthocyanin content for the both treatment of pectinase and protease enzyme, as well as for the treatment using the mixture of the enzymes. The same for antioxidant capacity, it's not affected by overall treatment, since the major antioxidant capacity of pomegranate juice is due to phenolic compounds and anthocynin.

Table 1. Effect of pectinase and protease enzyme concentration on the characteristics of pomegranate juice.

| | Untreated | Pectinase | | | Protease | | | 0.25 % Protease + 0.25 % pectinase |
|---|---|---|---|---|---|---|---|---|
| | | 0.05 | 0.25 | 0.5 | 0.05 | 0.25 | 0.5 | |
| pH | 4.15 ± 0.12 | 3.82 ± 1.21 | 3.77 ± 0.53 | 3.69 ± 0.47 | 3.87 ± 0.34 | 3.78 ± 0.61 | 3.72 ± 0.71 | 3.74 ± 0.27 |
| TSS (°Brix) | 15.7 ± 3.23 | 16.40 ± 5.12 | 16.60 ± 2.13 | 16.90 ± 1.52 | 16.20 ± 2.65 | 16.45 ± 1.53 | 16.82 ± 1.00 | 16.00 ± 1.00 |
| Titrable acidity | 0.25 ± 0.02 | 0.34 ± 0.11 | 0.31 ± 0.03 | 0.29 ± 0.05 | 0.35 ± 0.02 | 0.31 ± 0.04 | 0.30 ± 0.00 | 0.34 ± 0.05 |
| Maturity index | 62.80 ± 4.36 | 48.23 ± 7.45 | 53.54 ± 4.56 | 58,27 ± 5.00 | 46,28 ± 3.54 | 53,06 ± 6.04 | 56,06 ± 5.54 | 47,05 ± 6.34 |
| Total phenolic | 442.73 ± 32.12 | 447.12 ± 23.33 | 440.53 ± 52.00 | 432.23 ± 43.29 | 445.45 ± 27.56 | 438.67 ± 37.12 | 432.75 ± 13.00 | 491.34 ± 28.12 |
| anthocyanin content | 15.51 ± 3.02 | 15.28 ± 2.32 | 14.91 ± 2.22 | 14.56 ± 3.07 | 15.41 ± 2.06 | 15.01 ± 1.12 | 14.97 ± 2.82 | 15.81 ± 1.67 |
| antioxidant capacity | 51.75 ± 1.88 | 51.05 ± 2.38 | 50.67 ± 3.67 | 50.55 ± 3.64 | 51.15 ± 1.88 | 50.55 ± 1.88 | 50.13 ± 1.88 | 51.89 ± 5.88 |
| Total pectin | 2.37 ± 0.32 | 2.34 ± 1.01 | 2.20 ± 0.15 | 2.23 ± 0.21 | 2.15 ± 0.66 | 2.04 ± 0.11 | 2.36 ± 0.57 | 2.12 ± 0.43 |
| Total protein | 268.61 ± 18.23 | 264.51 ± 31.22 | 257.51 ± 12.26 | 252.51 ± 32.13 | 261.12 ± 46.00 | 251.51 ± 11.26 | 255.61 ± 12.26 | 253.43 ± 23.15 |
| Clarity | 0.062 ± 0.001 | 0.048 ± 0.005 | 0.032 ± 0.001 | 0.021 ± 0.003 | 0.053 ± 0.007 | 0.041 ± 0.002 | 0.029 ± 0.001 | 0.018 ± 0.001 |
| Turbidity | 115.32 ± 5.34 | 70.20 ± 3.65 | 62.34 ± 11.45 | 32.33 ± 2.45 | 72.34 ± 5.56 | 65.22 ± 1.56 | 38.45 ± 4.43 | 32.45 ± 4.67 |

Total soluble solids (TSS) was expressed as °Brix, Titrable acidity as g of citric acid /100 mL, total phenolic as mg gallic acid equivalents/ 100 mL, anthocyanin content as mg cyanidin-3-glucoside/ 100 mL, Antioxidant activity as percentage of Trolox inhibition; pectin as mg D-galacturonic acid equivalents/L, protein as g bovine serum albumin/L, clarity as Absorbance at 660 nm and turbidity as Nephelometric Turbidity Units (NTU). All measurements were done in triplicate

## Conclusion

The clarity of the juice is an important factor regarding the quality of the juice as it fetches consumer attention for the product in the market. By comparing the reduction of turbidity of juice after enzymatic treatment by pectinase and protease, pectinase of 0.5% v/v concentration improved the overall quality of pomegranate juice compared to protease enzyme treatment. The mixture of pectinase and protease increased the juice clarity. The main implication of the results obtained is that protease addition may be a workable alternative measure for decreasing the immediate turbidity levels of pomegranate juice and that pectinase addition may be relevant for keeping turbidity in check during cold storage of pomegranate juice.

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## REFERENCES

ALVAREZ S, ALVAREZ R, RIERA FA AND COCA J. **Influence of depectinization on apple juice ultrafiltration**. *Colloids and Surfaces A: Physicochemical and Engineering 1998,* 138: 377–382.

BENZIE IF. **Evolution of antioxidant defence mechanisms**. *European Journal of Nutrition* , 2000, 39: 53–61.

BRAND-WILLIAMS, W., M. CUVELIER AND C. BERSET,. **Use of a free radical method to evaluate antioxidant activity.** *LWT-Food Science and Technology, 1995,* 28: 25-30

CECI L AND LOZANO J. **Determination of enzymatic activities of commercial pectinases for the clarification of apple juice**. *Food Chemistry, 1998,* 61: 237–241.

EL NEMR SE, ISMAIL IA AND RAGAB M. **Chemical composition of juice and seeds of pomegranate fruit.** *Molecular Nutrition* and *Food Research* , 1990,34: 601–606.

FARIA A, MONTEIRO R, MATEUS N, AZEVEDO I AND CALHAU C. **Effect of pomegranate (*Punica granatum*) juice intake on hepatic oxidative stress**. *European Journal of Nutrition, 2007,* 46: 271–278.

FILISETTI-COZZI TMCC AND CARPITA NC. **Measurement of uronic acids without interference from neutral sugars.** *Analytical Biochemistry, 1991,* 197: 157–162.

GIL MI, TOMAS-BARBERAN FA, HESS-PIERCE B, HOLCROFT DM AND KADER AA. **Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry, 2000,* 48, 4581–4589.**

GRASSIN C AND FAUQUEMBERGUE F. **Fruit juices**, In: Godfrey T, West S and West S. (eds) Industrial Enzymology, second Ed., 1996, 225–264, Stockholm Press, New York.

GUYER DE, SINHA NK, CHANG TS AND CASH JN. **Physicochemical and sensory characteristics of selected Michigan cherry cultivars**. *Journal of Food Quality, 1993,* 16: 355–370.

KILARA A. **Enzymes and their uses in the processed apple industry:** a review. *Process Biochemistry, 1982,* 23: 35–41.

LANDBO A-K, PINELO M, VIKBJERG A, LET M AND MEYER AS. **Protease-assisted clarification of black currant juice: synergy with other clarifying agents and effects on the phenol content.** *Journal of Agricultural and Food Chemistry, 2006,* 54: 6554–6563.

LOWRY OH, ROSENBROUGH NJ, FARR AL AND RANDALL RJ. **Protein measurement with the Folin Phenol reagent**. *Journal of Biological Chemistry, 1951,* 193: 265-275.

OZGEN, M., C. DURGAÇ, S. SERÇE AND C. KAYA, **Chemical and antioxidant properties of pomegranate cultivars grown in the Mediterranean region of Turkey**. *Food chemestry*.,2008, 111: 703–706

SIEBERT KJ. **Haze formation in beverages**. *LWT-Food Science and Technology, 2006,* 39: 987–994.

SINGLETON VL AND ROSSI J. **Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents**. *American Journal of Enology and Viticulture, 1965,* 16: 144–158.

TUNG-SUN C, SIDDIQ M, SINHA N AND CASH J. **Commercial pectinase and the yield and quality of stanley plum juice**. *Journal of Food Processing and Preservation, 1995,* 19: 89–101.

VAILLANT F, MILLAN A, DORNIER M, DECLOUX M AND REYNES M. **Strategy for economical optimisation of the clarification of pulpy fruit juices using crossflow microfiltration**. *Journal of Food Engineering, 2001,* 48: 83-90.

VAILLANT F, MILLAN P, O'BRIEN G, DORNIER M, DECLOUX M AND REYNES M. **Crossflow microfiltration of passion fruit juice after partial enzymatic liquefaction.** *Journal of Food Engineering, 1999,* 42: 215–224.

YUSOF S AND IBRAHIM N. **Quality of soursop juice after pectinase enzyme treatment**. *Food Chemistry* , 1994,51: 83–88.