

# Peroxidase and polyphenoloxidase activity in tomato *in natura* and tomato purée

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**ABSTRACT.** Oxidative enzymes are responsible for changes in flavor, texture, color and also in some nutritive properties of several fruits and vegetables processed in industries. The present investigation aimed to study the activity of Peroxidase (E.C. 1.11.1.7) and Polyphenoloxidase (E.C. 1.10.3.1) in tomato fresh pulp *in natura* and in three different trademarks of tomato purée sold in supermarkets. Crude extracts of peroxidase (POD) and polyphenoloxidase (PPO) enzymes, from both fresh tomato pulp *in natura* and processed tomato purée, purchased in supermarkets, were prepared, by using sodium phosphate buffer at different pHs. The best pH values for extraction of peroxidase and polyphenoloxidase were 6.2 and 7.5, respectively. Protein precipitations were carried out using acetone at the ratio of 1:2 enzymatic extract/acetone. The enzymatic extract from Santa Clara tomato cultivar was concentrated 10 times, before being applied to the chromatographic column packed with Sephacryl 100-HS. From the samples eluted, one isoenzyme was isolated ( $A_1$ ) and the molecular weight and isoelectric point (pI) was determined (MW 45 kDa and pI 4.5). The fraction with  $A_1$  was stored at  $-18^\circ\text{C}$ , and later used for thermostability studies. A decrease in the enzymatic activity was observed at 85 and  $90^\circ\text{C}$ , in the enzymatic extracts obtained from fresh tomato pulp; nevertheless, in the processed tomato purées the loss of activity was more significant.

**Key words:** tomato, oxidative enzyme, isoenzyme, isolation, thermostability.

**RESUMO. Atividade de peroxidase e polifenoloxidase em tomate ‘in natura’ e purê de tomate.** Enzimas oxidativas contribuem para mudanças no sabor, textura, coloração e algumas propriedades nutricionais de frutos e vegetais processados. O presente trabalho teve por objetivo estudar a atividade da Peroxidase (E.C. 1.11.1.7) e da Polifenoloxidase (E.C. 1.10.3.1) em polpa de tomate ‘in natura’ e em três diferentes marcas de purês de tomate industrializados. Extratos brutos de peroxidase (POD) e polifenoloxidase (PPO) de tomate ‘in natura’ e de purê de tomate foram preparados usando tampão fosfato de sódio, com diferentes pHs. O melhor pH de extração, encontrado para a peroxidase e a polifenoloxidase foi 6,2 e 7,5, respectivamente. Para a precipitação de proteínas foi utilizado acetona, na proporção 1:2 de extrato enzimático/acetona. O extrato enzimático da polpa de tomate da cultivar Santa Clara foi concentrado dez vezes antes de ser aplicado na coluna cromatográfica empacotada com Sephacryl 100-HS. Das frações eluídas, uma isoenzima ( $A_1$ ) foi isolada, e a massa molecular e ponto isoelétrico (pI) foram determinados (PM 45 Kda e pI 4,5). A fração contendo a isoenzima ( $A_1$ ) foi armazenada a  $-18^\circ\text{C}$  e, posteriormente, foi utilizada para os estudos quanto à estabilidade térmica. Observou-se um decréscimo na atividade enzimática, nas temperaturas de 85 e  $90^\circ\text{C}$ , nos extratos obtidos do tomate ‘in natura’, no entanto nos purês o decréscimo da atividade foi mais acentuado.

**Palavras-chave:** tomate, enzimas oxidativas, isoenzima, isolamento, termo-estabilidade.

## Introduction

Peroxidase (POD) and polyphenoloxidase (PPO) are enzymes found abundantly in fruits and vegetables. Many studies (ROBINSON, 1991; KHAN; ROBINSON, 1993; DU; BRAMLAGE, 1995; CLEMENTE; PASTORE, 1998; LUÍZ et al., 2007) describe the enzyme action on substances,

which show bright colors due to oxidation. The action of PPO and POD is connected with the enzymatic darkening and loss of flavor when fruits and vegetables undergo industrialization processes to be turned into canned and/or frozen products (CLEMENTE, 1993). The pigment instability, in canned products, is one of the major undesirable phenomena that appear during food processing. It

occurs due to the action of PPO and POD enzymes, which remain active even after being submitted to the usual heating process of treatment (LÓPEZ-SERRANO; ROS BARCELÓ, 1996).

The reduction of deterioration reactions is one of the main objectives of the food industry (PECHER et al., 1990). During the processing or industrialization of tomatoes, lipoxygenase (LPO), peroxidase (POD) and polyphenoloxidase (PPO) enzymes can cause deterioration in the product, thus changing characteristics, such as color, aroma composition and nutritional properties of the product, especially causing a reduction of provitamin A (ESKIN, 1990).

Tomato sauce is the most important seasoning used around the world. Tomato purée possesses at least 18% (w v<sup>-1</sup>) of drying substances, apart from sodium chloride. In the present investigation, the processed tomato purée suffers a thermal treatment similar to the fresh tomato pulp treatment, with the objective of inactivating the oxidative enzymes, which are present in the product. The aim of the present investigation was to study the relationship between peroxidase and polyphenoloxidase action in fresh tomato pulp *in natura* and in tomato purée processed by food industries.

## Material and methods

Samples of fresh tomato pulp and processed tomato purée were analyzed in the present investigation: one sample of fresh tomato pulp *in natura* and three samples of tomato purée processed by three different food industries.

The ripe tomatoes *in natura* (Santa Clara Cultivar), used in the experiment, were supplied by a local producer. Tomatoes were taken to the Food Biochemistry Laboratory/UEM 12h after being picked and only perfect tomatoes were selected as sample to perform the assays. The cans of tomato purée, from three different food processing industries (designated A, B, and C), were purchased from a local supermarket. All chemical products of analytical grade used were obtained from BDH Light Laboratories Ltd.

## Extracts preparation

In order to extract polyphenoloxidase (PPO) and peroxidase (POD) from the fresh tomato pulp and from the processed tomato purée, enzymatic extracts were prepared by using 150 g of both fresh tomato pulp and processed tomato purée. The mixture was homogenized with 150 mL sodium phosphate buffer 100 mM, at different pHs (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 7.7, 8.0). Afterwards, the extracts were

centrifuged at 12,000 rpm, for 20 minutes, at 4°C. After being centrifuged the supernatants were named 'enzymatic extracts' and were classified with numbers from 1 to 8 and then stored at 18°C, for later analysis.

## Enzymatic activity

The method used to determine the peroxidase activity (POD) was based on the method described by Clemente (1998). The reaction mixture contained 2.7 mL of 0.03% H<sub>2</sub>O<sub>2</sub>, in 100 mM of sodium phosphate buffer, at pH 6.2 and 0.2 mL of POD extract sample. The enzymatic reaction was initiated by addition of 0.1 mL 1% (w v<sup>-1</sup>) of o-dianisidine solution in methanol. The initial change in absorbance was recorded at 460 nm, at 25°C, by using UV-VILE spectrophotometer (Hitachi U-200), for a period of 1 minute. Each sample was assayed in triplicate. A POD unit was defined as an increase in absorbance unit minute<sup>-1</sup> mL<sup>-1</sup>.

The polyphenoloxidase (PPO) activity was measured by using a modified spectrophotometric method (FUJITA et al., 1995), which is summarized as follows: The reaction mixture consisted of 0.5 mL of PPO extract; 0.8 mL sodium phosphate buffer 100 mM, pH 7.5; and 0.05 mL of catechol solution 10 mM. The mixture was then incubated for 30 minutes, at 30°C. Afterwards, 0.8 mL of perchloric acid solution 2 M was added, and then the tubes were immersed in ice bath. After that, PPO activity was measured ( $\lambda = 395$  nm). A POD unit was defined as an increase in absorbance unit per minute mL<sup>-1</sup>.

## Concentration of enzymatic extracts

The enzymatic extracts from both, the fresh tomato pulp and from processed tomato purée were concentrated before being applied to gel filtration chromatography. The proteins were precipitated using cold acetone at the proportion of 1 to 2 (enzymatic extract acetone<sup>-1</sup>). The precipitate was resuspended into a sodium phosphate buffer 100 mM (at the same pH the enzymatic extract was prepared), in a volume ten times smaller, in relation to the initial volume of the enzymatic extract (VADERRAMA; CLEMENTE, 2004).

## Chromatography

Before being submitted to the chromatography column, the POD concentrated extract was dialyzed (in sodium phosphate buffer 10 mM, at pH 6.2, for 12 hours, at 7°C), and then has undergone a filtration process by using a micro filter (0.22  $\mu$ m), in order to prevent small particles from blocking the filters in the column. Afterwards, the sample (5 mL)

was applied to a chromatographic column packed with Sephacryl HS-100 (i. d. 16 mm x 70 cm), which was pre-balanced with 100 mM phosphate buffer, at the respective pH, and was used as an elution solution. The flow rate used was 30 mL h<sup>-1</sup>, whereas the volume of each fraction was 5 mL. That stage was carried out in order to eliminate other molecules that could interfere in the thermal inactivation of enzymes. The fractions, which were eluted from the chromatographic column, and that showed enzymatic activity, were stored at -18°C to be used later in the thermal treatment.

The molecular weight of the isolated isoenzyme was estimated by gel-filtration (Sephacryl S-100 HR, a crossed linked dextran). It has been well documented that for a homologous series of compounds, there is a logarithmic relationship between the elution volume and the logarithm of its molecular weight. A calibration curve was prepared by measuring the elution volume (*Ve*) of standard low molecular weight proteins (Blue dextran 2000 MW 2,000,000; Bovine serum albumin MW 67,000; Ovalbumin MW 43,000; Chymotrysinogen MW 25,000 and Ribonuclease MW 13,000), and plotting the ratio *Ve/Vo* against the logarithm of molecular weights. The elution volume (*Ve*) was measured from the start of the sample application to the center of the elution peak monitored at 280 nm, as determined by the intersection of the two tangents drawn to the sides of the peak. Blue dextran 2000 was used to determine the void volume (*Vo*) (CLEMENTE, 1993).

#### Thermostability

Samples of the enzymatic extract, and also isoenzyme (POD), eluted from the gel filtration chromatography that presented enzymatic activity were submitted to thermal treatments at 80, 85, 90, 95°C, for periods of 1-12 minutes. The residual activity of PPO and POD was determined after the thermal treatments, as previously described.

#### Measurement of pH gradient

The pH gradient across the gel was measured using a surface electrode. In addition, a narrow strip of focused gel was cut into 5 mm pieces parallel with electrode strips. Each piece was soaked in 2 mL of distilled water for two hours before the pH of each resulting was measured (CLEMENTE, 1993).

#### Staining of gel plates

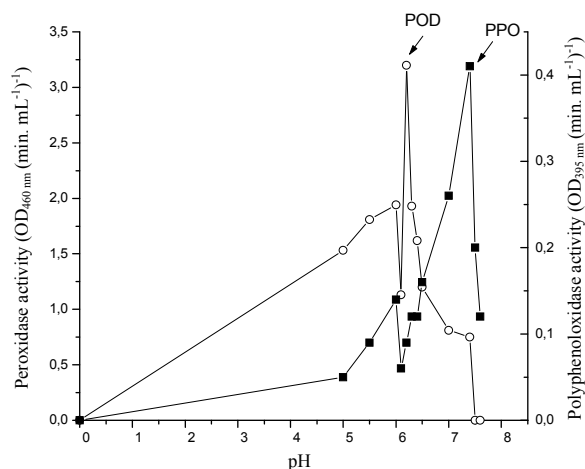
Following isoelectric focusing, the plate was specifically stained to show up peroxidase bands. The method used was adopted from that described by McLellan and Robinson (1987). It involved

immersing the gel plate in 150 mL of 100 mM sodium phosphate buffer at pH 6.2, followed by the addition of 32 mL of methanol solution and 8 mL of 0.1% (w v<sup>-1</sup>) o-dianisidine in methanol. The plate was then left to stand for 5 min. The staining reaction was then initiated by addition of 20 mL 0.3% (w v<sup>-1</sup>) H<sub>2</sub>O<sub>2</sub> and allowed to proceed for 30 min. at room temperature. Peroxidase activity showed up as brown bands on a clear background.

## Results and discussion

#### Enzymatic activities

Peroxidase (POD) and polyphenoloxidase (PPO) enzymatic activities at different pHs are shown in Figure 1.



**Figure 1.** Peroxidase (POD) and polyphenoloxidase (PPO) enzymatic activities in extracts obtained from fresh tomato pulp *in natura* at different pHs.

Such evaluation is considered necessary because there are many studies in literature, showing the most varied pH values to extract (POD) and (PPO) enzymes such as: from cabbage leaves pH 5.6 (ROLING et al., 2000); sweet potato pH 6.0 (BELINI et al., 2001); broccoli pH 6.0 (LOPES; CLEMENTE, 2002); uvaia fruit pH 6.5 (CLEMENTE; COSTA, 2006). These results showed that the variety of values for pH extraction, are influenced, not only by different cultivars, but also, by the way the crops are managed, by the local those enzymes are within the cell and by the way they are settled down (ionically or covalently), and also by another aspect: if they are soluble or not.

Prabha and Patwardhan (1986) reported that 7.4 was the best pH for PPO extraction from ripens fruits, such as mango, sapota and apples. Arnao et al. (1990), on the other hand, extracted peroxidase from tomato with pH 6.3; Clemente and Robinson (1995)

extracted peroxidase from orange cell walls, and also, from intracellular spaces and the best pH found for extraction of POD was 6.0. However, Cano et al. (1995) made the extraction of PPO and POD from papaya, during post harvest ripening and also after freezing it, using pH 7.0; whereas Roling et al. (2000), in their work resulting from cabbage experiments, extracted POD by using pH 6.0. Nevertheless, Troiani et al. (2003), when evaluating the activity of PPO and POD in grapes, carried out the extraction of those enzymes at pH 6.0. Carvalho and Clemente (2004) found pH 6.0, and stated that pH 6.0 is the best one for POD extraction from broccoli, whereas Laurenti and Clemente (2005) in their study with star fruit found the pH 7.0, regardless of the ripening stage of the fruit. The present study regarding pH was done to obtain information on the best pH index to extract the enzymes POD and PPO. The results found in the present investigation for tomato fresh pulp and processed purée (POD 6.2 and PPO 7.5) are within the pH ranges found in literature. However, before carrying out the extraction it is suggested doing an evaluation to isolate and purify those enzymes, because the isoenzymes composition can have a different amino-acid sequence, what would change their characteristics, for example, their thermal behavior.

In the next step, a new enzymatic extract from fresh tomato pulp and processed tomato purée was carried out, for POD and PPO, using sodium phosphate buffer 100 mM, at their respective pHs (6.2 and 7.5, respectively). The procedure was the same, as previously described. Results, regarding the enzymatic activities are shown in Table 1.

**Table 1.** Peroxidase and polyphenoloxidase activity mean in tomato pulp and tomato purée (n = 3).

| Sample | POD                                                  | PPO                                                  |
|--------|------------------------------------------------------|------------------------------------------------------|
|        | $\Delta OD_{405}^*$ (min. mL $\pm$ Sd) <sup>-1</sup> | $\Delta OD_{395}^*$ (min. mL $\pm$ Sd) <sup>-1</sup> |
| Tomato | 3.20 $\pm$ 0.01                                      | 0.41 $\pm$ 0.02                                      |
| A      | 0.38 $\pm$ 0.02                                      | 1.52 $\pm$ 0.03                                      |
| B      | 0.44 $\pm$ 0.01                                      | 2.32 $\pm$ 0.02                                      |
| C      | 0.38 $\pm$ 0.01                                      | 1.72 $\pm$ 0.01                                      |

\* $\Delta OD$  = change in optical density, Sd = standard deviations, n = number of measurements.

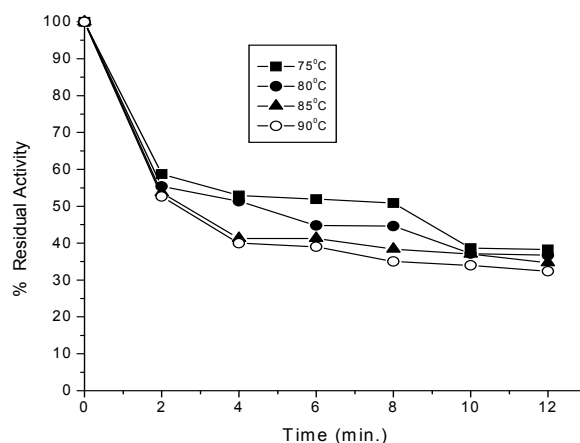
POD activity in the enzymatic extract from fresh tomato pulp was higher than those found in the enzymatic extracts taken from the processed tomato purée. The decrease of activity in tomato purée also shows that the pasteurization process carried out in food processing industries was enough to inactivate POD partially; but the remaining activity still causes a decrease in the product quality (CLEMENTE; PASTORE, 1998).

PPO activity showed inverse behavior – that is, a

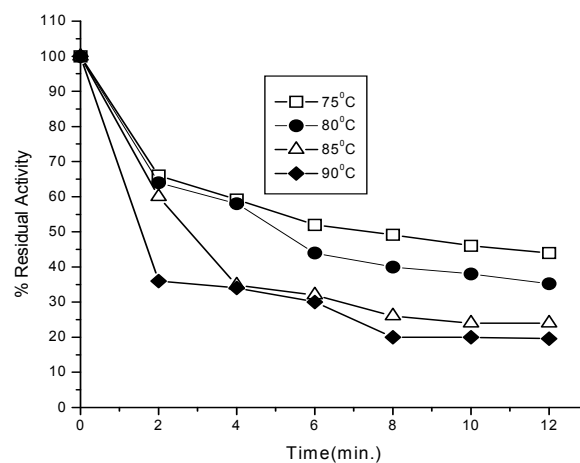
higher activity in the extracts was obtained from tomato purée. Perhaps, the transformation of tomato pulp into concentrated purées increased the concentration of this enzyme and the pasteurization process, used by industries, has not contributed incisively for PPO enzyme inactivation. These results also suggest a difference in the isoenzyme composition which presented a different behavior when exposed to the thermal treatment.

### Thermostability

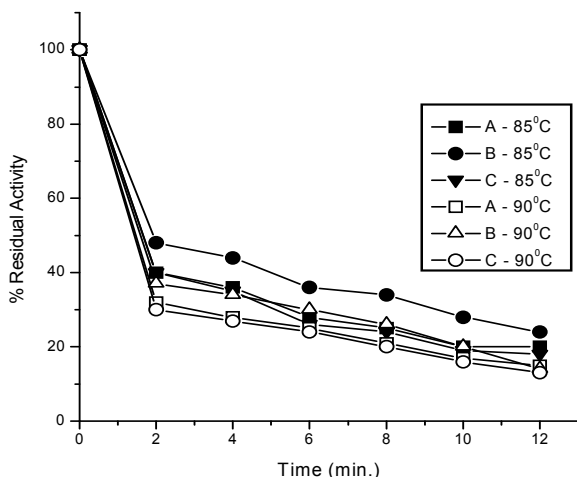
The enzyme extracts were submitted to thermal treatments at different temperatures (75, 80, 85 and 90°C) and different periods of time (0, 2, 4, 6, 8, 10 and 12 minutes). The results of POD remaining activity in fresh tomato are in Figure 2; whereas PPO results, also obtained from fresh tomato pulp, are in Figure 3. POD and PPO resulting activity, after the thermal treatments applied to the extracts obtained from processed tomato purée are shown in Figures 4 and 5, respectively.



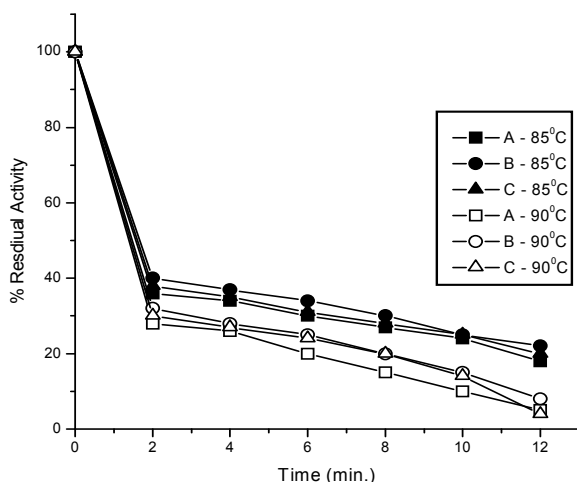
**Figure 2.** Remaining activity of peroxidase (POD) in fresh tomato pulp after thermal treatment from 75 to 90°C.



**Figure 3.** Remaining activity of polyphenoloxidase (PPO) in fresh tomato pulp after thermal treatment from 75 to 90°C.



**Figure 4.** POD residual activity in the three processed tomato purée A, B and C after thermal treatment at 85 and 90°C.



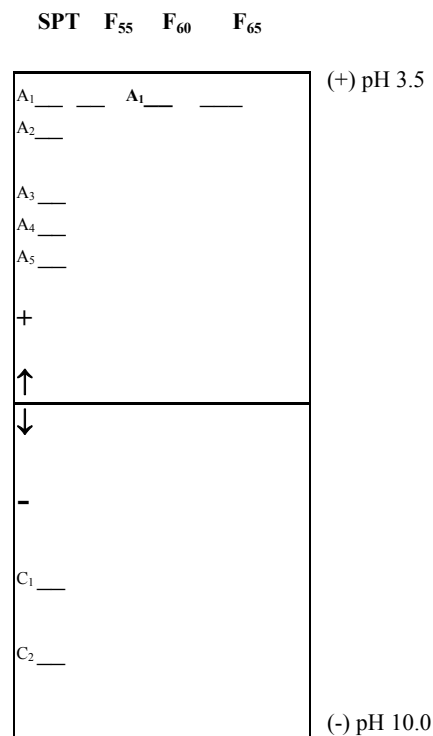
**Figure 5.** PPO residual activity of A, B and C processed tomato purée after thermal treatment at 85 and 90°C.

These results are in agreement with other investigations reported in literature, stating that fruit maturation stage cause an alteration in these enzymes activity, the enzyme activity is not totally eliminated when the product undergoes industrial process which uses high temperature and short time (HTST) (PRABHA; PATWARDHAN, 1986; KHAN; ROBINSON, 1993; RESENDE et al., 2004; LAURENTI; CLEMENTE, 2005; GONÇALVES et al., 2006; CLEMENTE; COSTA, 2006). The remaining enzyme activity will continue affecting product shelf life.

#### Chromatography and isoelectric focusing

It was possible to isolate an anionic isoenzyme defined as A<sub>1</sub> by using chromatographic column packed with Sephacryl S-100 HR. Results regarding isoelectric focusing is shown in Figure 6. Other isoenzymes, present in the enzymatic fraction of POD and obtained from fresh tomato pulp, can be

observed in Figure 6. The presence of five anionic and two cationic isoenzymes were clearly seen in the sample from enzymatic extracts obtained from tomato pulp. However, it was not possible to isolate all of them, because, probably, some of them were missing during the chromatographic process and also because the dilution made difficult to detect them.



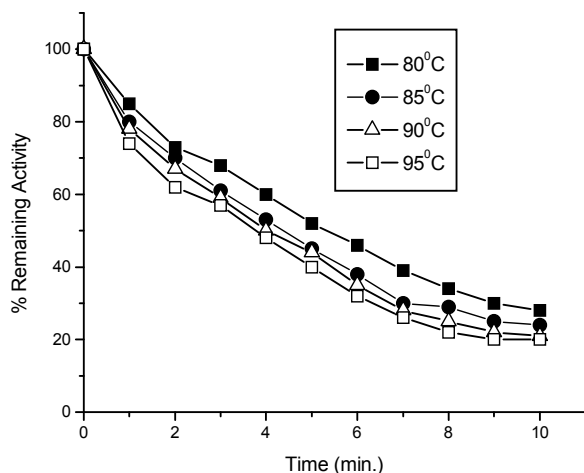
**Figure 6.** Electrophoretic patterns diagram obtained for fractions, after fractionation by Sephacryl S-100 HR column. Sample taken from POD fresh tomato pulp extract (SPT – soluble POD from fresh tomato pulp, F – fraction eluted from column).

The equation showing the calibration curve of the standard molecular weight used for determining the molecular weight of isolated isoenzyme was ( $Y = 2.3452 - 0.4533X$ ) where  $Y = K_{av}$  and  $X = \log$  (molecular weight),  $K_{av} = V_e - V_o / V_t - V_o$   $K_{av}$  is the elution parameter;  $V_e$  is the elution volume for the protein;  $V_o$  is the column void volume which is the elution volume for Blue Dextran; and  $V_t$  is the total volume. The isolated anionic isoenzyme (A<sub>1</sub>) presented a molecular weight of approximately 45 kDa and pI 4.5.

Thus, the value obtained for the anionic isoenzyme MW was similar to the isoperoxidase found in kiwifruit, MW 40 kDa – 42 kDa (PRESTAMO, 1989); peanuts MW 40 kDa – 42 kDa (HU et al., 1989); and orange MW 43 kDa – 44 kDa (CLEMENTE, 1998).

The isolated isoenzyme was then submitted to the thermal treatment at temperatures that varied

from 80 to 95°C. The result of such enzymatic behavior, in the thermal treatment, can be observed in Figure 7. Results show that the decline of the enzymatic activity is non-linear. Results are in agreement with reports by McLellan and Robinson (1984); Khan and Robinson (1993); Valderrama and Clemente (2004). However, 10 minutes later, at 95°C temperature, the isoenzyme maintained 20% of its initial activity. Such a finding is similar to other ones reported in literature focusing peroxidase isoenzymes. It corroborates findings by Clemente (1998) in his studies on thermostability of isolated isoenzymes in orange.



**Figure 7.** Isoenzyme activity ( $A_1$ ) from fresh tomato pulp versus time, heated up at 80, 85, 90 and 95°C.

## Conclusion

A larger enzymatic inactivation was observed mainly during the first four minutes at the temperatures of 85 and 90°C for both enzymes POD and PPO in all the samples extracts.

In the processed tomato purée samples, it could be noticed that the industrial food process used was clearly inefficient to inactivation of the enzymes.

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