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Inactivation of papain by pulsed electric fields in a continuous system

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Abstract

Papain (E.C. 3.4.22.2), a cysteine protease in papaya, was activated with reducing agents and treated with pulsed electric fields (PEF) in a continuous system at 10°C. Irreversible reduction of activity was observed in PEF-treated papain after 24 h storage at 4°C. Oxidation of papain active site, a cyteine residue, was not the major cause of papain inactivation by PEF. Temperature did not increase over 35°C during PEF treatment. The pH of papain solution was not changed after PEF treatment. Structural change was observed in PEF-treated papain by CD analysis. Inactivation of PEF-treated papain was related to the loss of α-helix structure. Heating at 60-80°C for 2 min did not significantly reduce the activity of papain.

1. Introduction

High voltage pulsed electric fields (PEF) is a nonthermal food preservation method studied for 30 years. Many studies focus on the inactivation of microorganisms by PEF (Castro, Barbosa-Canovas, & Swanson, 1993; Hamilton & Sale, 1967; Hulsheger & Niemann, 1980; Marquez, Mittal, & Griffiths, 1997; Sale & Hamilton, 1967; Zhang, Monsalve-Gonzalez, Qin, Barbosa-Canovas, & Swanson, 1994; Zhang, Qin, Barbosa-Canovas, & Swanson, 1995).

In comparison with the extensive research devoted to the destruction of microorganisms by PEF, there are few reports about the inactivation of enzymes by PEF. Vega-Mercado, Powers, Canovas and Swanson (1995) reported the reduction of plasmin (milk alkaline protease) activity up to 90% in a simulated milk ultrafiltrate using a closed-loop system after 50 pulses at either 30 or 45 kV/cm. Grahl and Markl (1996) reported a 60% inactivation of lipase in raw milk at 21.5 kV/cm using a batch treatment chamber. Ho, Mittal, and Cross (1997) reported that the effects of high voltage pulses on activities of eight enzymes varied from enzyme to enzyme. To elucidate enzyme inactivation mechanism, information about enzyme structure is necessary. Although a few food enzymes were inactivated by PEF, mechanisms involved in inactivation of enzymes by PEF are not fully understood due to the lack of enzyme structural data.

Papain (E.C. 3.4.22.2) was selected as a model enzyme to investigate the mechanism of enzyme inactivation by PEF. Papain is a major protein constituent of the latex in the papaya fruit and one of the most extensively studied food enzymes (Drenth, Jansonius, Koekoek, & Wolthers, 1971). Structure information of papain is available from the primary sequence to the tertiary structure (Baker & Drenth, 1984). The function of the secondary structure was reported by Lavery, Pullman, and Wen (1983). Papain is a relatively small protein having a molecular weight of 23,406. It is a single chain polypeptide of 212 amino acids and three disulfide bridges (Lowe, 1976). The simplicity of the papain structure makes it easy to understand changes in its activity and structure. PEF is a non-thermal processing method, however an increase in temperature (e.g. up to 30–40°C) can occur during PEF treatment depending on the sample composition and the processing condition. If the enzyme is heat-labile, it is difficult to distinguish any thermal effect from non-thermal effects of PEF on the enzyme. It is necessary to use a heat-resistant enzyme in the study of the mechanism of enzyme inactivation by PEF. Papain is heat stable (Schomburg & Salzmann,

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1991) and its heat stability was confirmed by thermal inactivation experiments.

2. Materials and methods

Papain was purchased from Boehringer Mannheim (Indianapolis, USA). L-cysteine, dimethyl sulfoxide, N_{α}-benzoyl-$_{DL}$ arginine ρ-nitroanilide (BAPNA), 2,2′-dithiodipyridine (2PDS), $_{DL}$-dithiothreitol ($_{DL}$-DTT) and Sephadex G-25 were obtained from Sigma (St. Louis, USA).

2.1. Preparation of activated papain

Active papain has a single free cysteine at its active site. During storage, papain is reversibly inactivated in the presence of air due to the formation of a mixed disulfide bond (Glazer & Smith, 1971). The reversibly inactivated papain can be activated by reducing agents such as cysteine or dithiothreitol (DTT). Addition of ethylenediaminetetraacetate (EDTA) is also required for maximum activity (Baines & Brocklehurst, 1982). For activation, papain was incubated in 20 mM L-cysteine for 30 min, then in 5 mM dithiothreitol (DTT) for 5 min (Fig. 1). To test the effects of PEF on papain, a suspension medium should be similar to a food system that does not usually contain the reducing agents. Accordingly, activated papain was separated from the reducing agents using Sephadex G-25 chromatography. After being separated from the reducing agents, papain was diluted with 1 mM EDTA. To know whether PEF inactivated papain by oxidation of papain active site, the reducing agents were included in PEF treatment. Papains for control and PEF-treatment were prepared from the same activated papain solution.

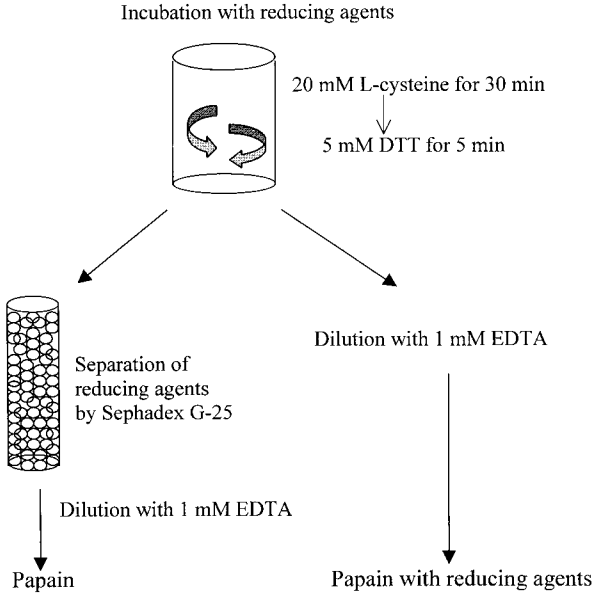

Fig. 1. Diagram of the papain activation. All procedures were performed at room temperature.

2.2. Determination of protein concentrations

Protein concentrations of papain were determined by a spectral analysis at 280 nm using Beer–Lambert's law (Harris, 1987):

$$A = \varepsilon bC$$

where A is absorbance, ε is extinction coefficient ($M^{-1}\,cm^{-1}$), C is concentration (M), and b is pathlength (cm). The value of ε_{280} for papain is 5.6×10^4 $M^{-1}cm^{-1}$ (Brocklehurst & Little, 1973).

2.3. Determination of papain activity

The activity of papain was measured by a spectral analysis at 410 nm using $_{DL}$-BAPNA as a substrate (Baines & Brocklehurst, 1982). A stock solution of DL-BAPNA was prepared by dissolving 43.5 mg of DL-BAPNA in 1 ml of dimethylsulfoxide and subsequently increasing the volume to 100 ml with 0.05 M Tris/HCl buffer, pH 7.5, containing 1 mM EDTA. The A_{410} of a sample cell containing 2 ml of substrate solution was balanced against that of a reference cell containing 2 ml of the Tris–HCl buffer in a Spectronic Genesys 5 spectrometer (Milton Roy, Rochester, USA) at room temperature. After 1 ml of papain was added to the reference cell, 1 ml of papain was added to the sample cell. The linear increase in absorbance was recorded for 5 min. The concentrations of ρ-nitroaniline, the chromophoric product, were calculated by Beer–Lambert's law using $\varepsilon_{410} = 8.8 \times 10^3$ M^{-1} cm^{-1} (Erlanger, Kokowsky, & Cohen, 1961). The relative activity of papain was calculated with the following formula:

$$\text{Specific activity}\,(\text{min}^{-1}) = \frac{\text{Release of product}: \rho\text{–nitroaniline}\,(\mu\text{M/min})}{\text{Papain concentration}\,(\mu\text{M})}$$

$$\text{Relative activity}(\%) = \frac{\text{Specific activity of papain after PEF or Heat treatment}}{\text{Specific activity of control papain}} \times 100$$

Control papain was circulated in PEF system under the same processing conditions without PEF treatments. All experiments were replicated according to the randomized complete block design. Analysis of variance (ANOVA) and Tukey multiple comparisons method were used for statistical analysis of data at the 5% level.

2.4. PEF treatment

A bench scale continuous PEF system (OSU-1) at the Ohio State University was used to treat the papain (Fig. 2). A Model 2829 series pulser (Cober Electronics Inc., Stamford, CT, USA) was used to supply the high voltage electrical pulses. Square wave pulses of voltage and current were monitored by a two channel 500 MS/s (100 MHz bandwidth) digital oscilloscope (Model TDS 320, Tektronix Inc., Beaverton, OR, USA). Co-field flow, tubular PEF treatment chamber (Yin, Zhang, & Sastry, 1997) provided the selected treatment condition. Total four PEF treatment chambers were used in this study. Two chambers were connected with stainless steel tubing as a pair of chambers. A cooling coil was connected to each pair of chambers and submerged in a water bath at 10°C. Type K thermocouples (Fisher Scientific, Pittsburgh, PA, USA) were attached to the surface of the cooling coils. Temperatures were monitored during PEF treatment by a dual channel digital thermocouple reader (Fisher Scientific, Pittsburgh, PA, USA). The pulse duration time (τ) was selected as 4 μs; the frequency (f) was 1500 pulses per; and the flow rate was 0.77 ml/s. The total volume of papain in the PEF system was maintained at 150 ml for all experiments.

2.5. Thermal inactivation of papain

Papain (2–3 μM, 1.2 ml) was placed in a 1.5 ml microcentrifuge tube (Fisher Scientific, Pittsburgh, PA, USA) and heated at a selected temperature (60–90°C) for 2 min in a water bath. A type K thermocouple with 0.08 inch of diameter was inserted in the center of the tube and the temperature of papain was monitored by a dual channel digital thermocouple reader (Fisher Scientific, Pittsburgh, PA, USA). The heating time was counted after the temperature of the papain reached the desired temperature. The tubes were removed after heating and cooled in ice bath until tested for activity.

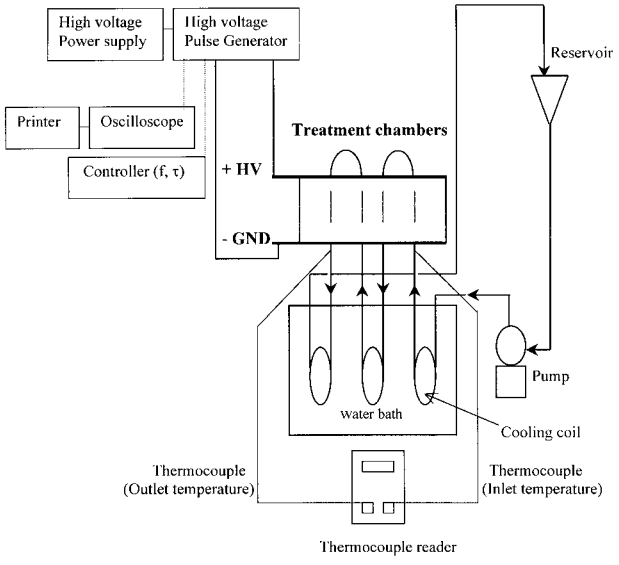

Fig. 2. Diagram of the PEF bench-scale processing unit (OSU-1).

2.6. Determination of free cysteine content in papain

Active papain has a single free cysteine at its active site. When papain is inactivated by the oxidation of its active site, the content of free cysteine in papain is decreased. To monitor the oxidation of active site, the content of free cysteine in papain was measured by a titration reaction with 2,2′-dipyridyl disulphide (2PDS). The concentration of 2-thiopyridine, the chromophoric product released in the titration reaction, was measured by a spectral analysis at 343 nm using Beer–Lambert's law (Shipton & Brocklehurst, 1978). The relative content of free cysteine was determined with the following formula:

$$\text{Content of free cysteine (\%)} = \frac{\text{Concentration of 2-thiopyridine (μM)}}{\text{Concentration of papain (μM)}} \times 100$$

$$\text{Relative content of free cysteine (\%)} = \frac{\text{Content of free cysteine in papain after PEF}}{\text{Content of free cysteine in control papain}} \times 100$$

2.7. CD analysis of papain

Circular dichroism (CD) can directly interpret the changes of protein secondary structures (Venyaminov & Yang, 1996). The CD analysis was used to detect change of secondary structure in papain after PEF treatment. CD spectra were scanned at the far UV range (260–200 nm) with a Jasco J-500 A spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan) and quartz CD cells (Hellma, Muellheim, Baden, Germany). The CD spectra of papain were measured at room temperature using 1 mM EDTA as a blank solution. The CD spectropolarimeter is designed to measure absorbance difference, ΔA, and it collects ΔA in terms of voltage (Chang, 1997). For peptides or proteins, CD data are usually expressed as "mean residue ellipticity" (Woody, 1996). To calculate the mean residue ellipticity ([θ], in units of deg cm^2 dmol^{-1}) from the collected data (V, volts), the following formula (Chang, 1997) was used:

$$[\theta] = \frac{V(\text{volts}) \times f \times 3298}{d \times C_M \times N_{AA}}$$

where V is the signal collected by spectropolarimeter (volts), f is the correction factor from calibration of

spectropolarimeter, d is path length (cm), C_M is molar concentration (M), and N_{AA} is number of amino acids in the papain.

3. Results and discussion

3.1. Inactivation of papain by PEF

Fig. 3 illustrates the effects of PEF on papain activity at 50 kV/cm. Analysis of variance (ANOVA) at the 5% level indicated that PEF had no effect on the papain activity at 0 h when the activity was measured immediately after PEF treatment. However, significant reduction of activity in PEF-treated papain was observed after 24 h storage at 4°C. T method at the 5% level indicated that there was a significant difference between two treatment groups, 200–300 pulses and 400–500 pulses. A higher number of pulses at 50 kV/cm caused higher reduction of papain activity. Similar results were obtained in PEF-treated papain at selected electric field strengths from 20 to 50 kV/cm (Fig. 4). After 24 h storage at 4°C, PEF-treated papain exhibited a significant decrease of activity regardless of the electric field strength.

3.2. Chemical oxidation of papain by PEF

A chemical oxidation was considered the cause of papain inactivation, because the active site of papain consists of a cysteine residue that is sensitive to oxidation. The relative content of free cysteine was measured in PEF-treated papain after 24 h storage at 4°C (Fig. 5). Decrease in the relative content of free cysteine was observed at 50 kV/cm but it was not consistent with the significant reduction of papain activity after 24 h storage. To know whether PEF inactivated papain by oxidation of papain active site, the reducing agents were included in PEF treatment. If papain was inactivated by PEF primarily due to the oxidation of its active site, the existence of reducing agents should prevent inactivation of papain. However, papain was also inactivated by PEF in the existence of reducing agents (Fig. 6). The

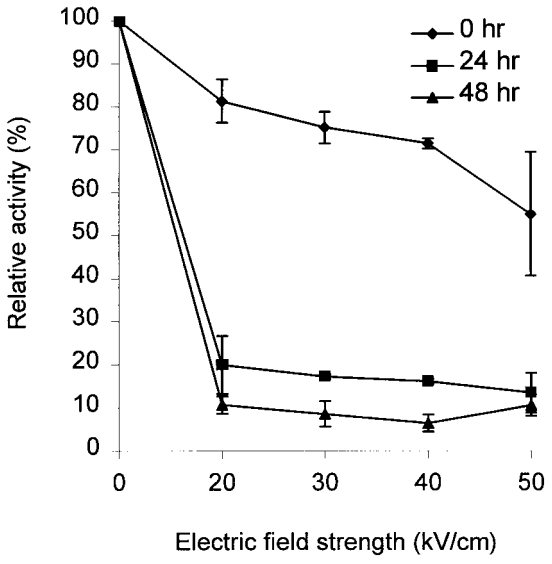

Fig. 4. Relative activity of PEF-treated papain during storage time at 4°C. Total treatment time was 2 ms, which is equivalent to 500 pulses.

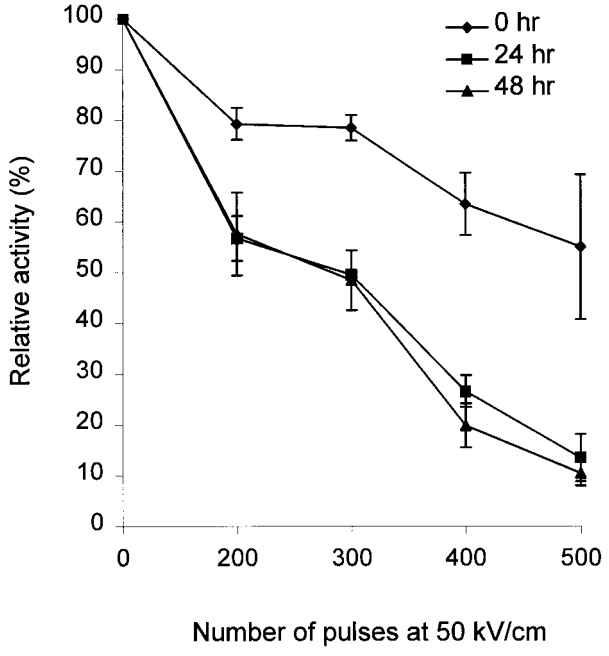

Fig. 3. Relative activity of PEF-treated papain during storage time at 4°C.

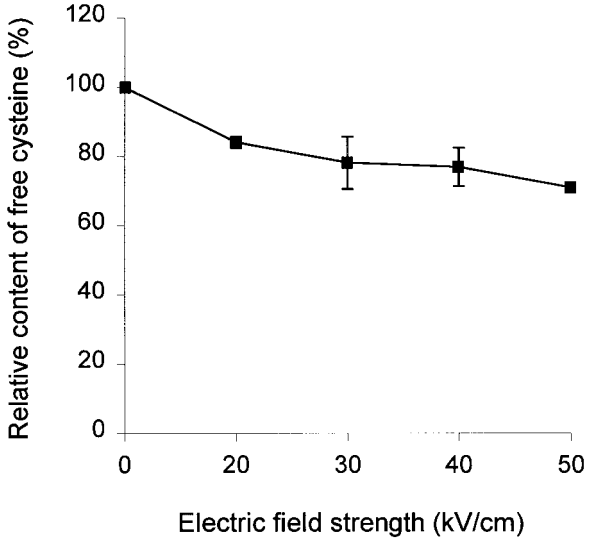

Fig. 5. Relative content of free cysteine in PEF-treated papain after 24 h storage at 4°C. Total treatment time was 2 ms, which is equivalent to 500 pulses.

PEF-treated papain with reducing agents was incubated with 20 mM of cysteine and 5 mM of DTT to reactivate oxidized active site of papain. After reactivation, only a 2–4% increase of papain activity was observed. The suspension medium, 1 mM EDTA, was treated with PEF to test if PEF produced any oxidizing compounds from the stainless electrodes of the treatment chamber. Papain was incubated with reducing agents for activation then divided into two separate bottles. The 150 ml of 1 mM EDTA was treated at 50 kV/cm with 500 pulses, then immediately added to one bottle containing papain. At the same time, untreated 1 mM EDTA was also added to the other bottle. After being diluted with PEF-treated 1 mM EDTA, papain showed 87.7 ±6% of relative activity. This value was higher than 44.1 ±8% of relative activity of PEF-treated papain at the same condition. The results indicate that the oxidation of papain active site is not the major cause of papain inactivation by PEF.

There was no change in the pH of papain solution after PEF treatment. The pH of control and PEF-treated papain solutions was 6.0 ± 0.5, which is within the stable range of pH 4–9 (Schomburg & Salzmann, 1991).

3.3. Thermal and shearing effects of PEF

Temperature did not increase over 35°C during PEF treatment. Papain was heat stable at 60–80°C in thermal inactivation experiments. Consequently, thermal inactivation was excluded from the papain inactivation mechanism of PEF.

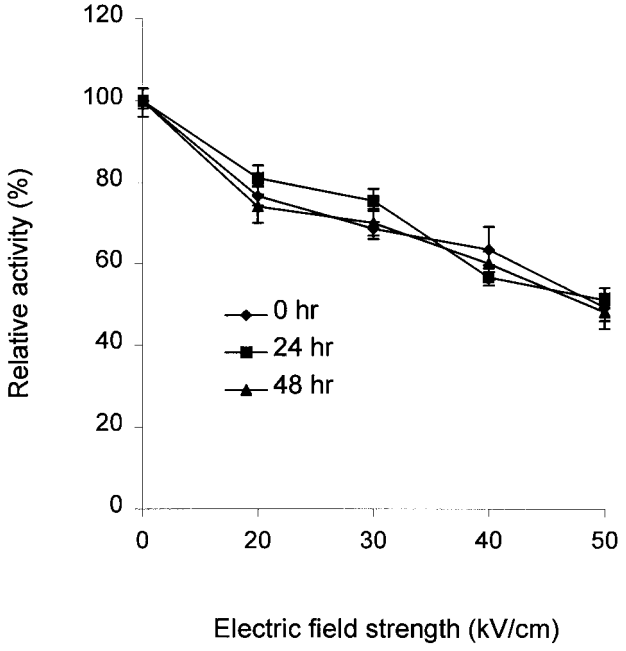

Fig. 6. Relative activity of PEF-treated papain with reducing agents during storage time at 4°C. Total treatment time was 2 ms, which is equivalent to 500 pulses.

Charm and Wong (1981) studied the shear effects on enzymes during flowing in tubes, pumping and mixing. A pump (Model HG030, Micropump Co., Vancouver, WA, USA) was used to process papain in a bench scale continuous PEF system. Control papain was circulated in the PEF system at the same conditions except electric pulse to test the shear effects. There was no decrease of papain activity at the flow rate of 0.77 ml/s.

3.4. Structural change of papain by PEF

Papain has double negative peaks in far UV CD spectra at 220 and 208 nm (Xiao, Liang, & Tsou, 1993), which are characteristic of an α-helix structure. Intensity of CD peaks at 222 and 208–210 nm reflects the amount of helical structure in protein (Venyaminov & Yang, 1996). The intensity of two negative peaks in CD spectra of papain decreased after PEF treatment at 50 kV/cm (Fig. 7). It indicated a loss of α-helix structure in papain after PEF treatment.

Dipole moment is defined as a vectorial quantity due to a pair of positive and negative charges (Wada, 1967). Each peptide bond carries a dipole moment. It is known that the alignment of the peptide bonds in the α-helix structure gives rise to a macrodipole of considerable strength (Wada, 1962, 1976). The electric field due to α-helix is used by proteins in binding charged groups and stabilizing transition states (Hol, Van Duijnen & Berendsen, 1978). Papain contains a long central α-helix and the N-terminal end of this helix is close to the active site (Van Duijnen, Thole, Broer, & Nieuwpoort, 1980; Van Duijnen, Thole & Hol, 1979). The function of the α-helix in papain is to help the proton transfer at papain active site (Baker & Drenth, 1984).

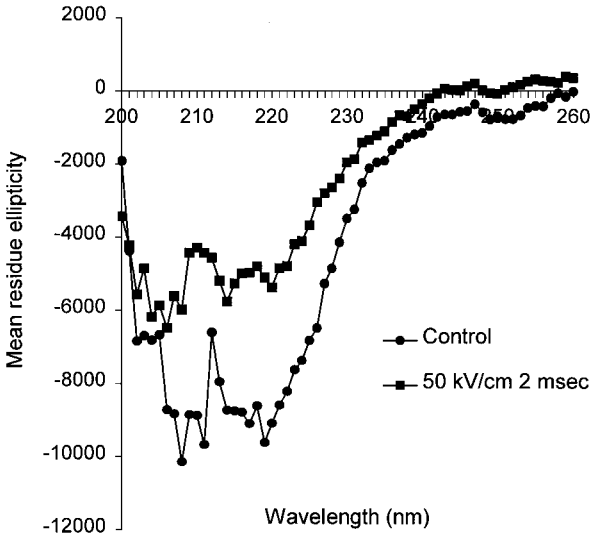

Fig. 7. CD spectra of control papain and PEF-treated papain at 50 kV/cm for 2 ms.

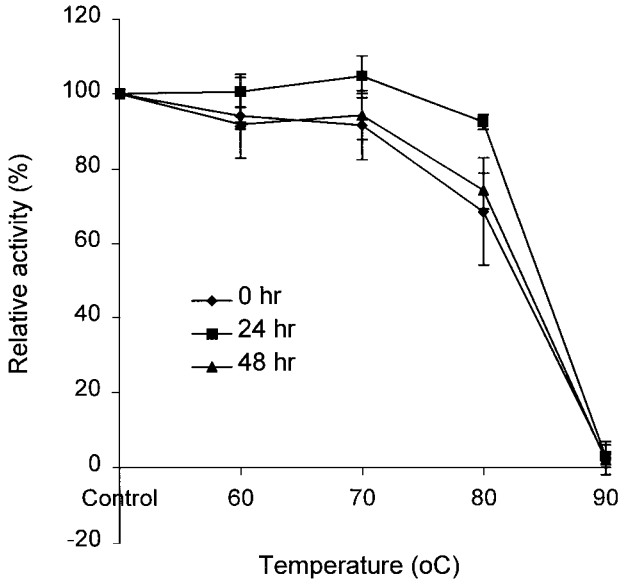

Fig. 8. Relative activity of heat-treated papain during storage time at 4°C. Total heating time was 2 min.

Electric fields can induce conformational changes in biopolymers and membranes (Neumann, 1986). Neumann and Katchalsky (1972) reported that electric pulses of about 20 kV/cm induced a long-lived helix–coil transition in polynucleotide helices. According to the calculation of Bean and Bennett (1973), electric fields of 30–300 kV/cm cause changes in the helix–coil transition temperatures of polypeptide chains. Helix-coil transition of synthetic polypeptides at 30 kV/cm was reported by Tsuji, Yasunaga, Sano, and Ushio (1976).

Papain contains α-helical structure, which is important in papain activity and susceptible to conformational change by high electric fields due to its dipole moment. Loss of α-helix structure was observed in PEF-treated papain with the significant reduction of papain activity. The inactivation of papain was related to the conformational change of α-helix induced by PEF.

3.5. Thermal inactivation of papain

Papain was significantly inactivated at 90°C (Fig. 8). The papain heated at 60–80°C for 2 min had greater activity than control papain after 24 h storage at 4°C.

4. Conclusion

Papain was irreversibly inactivated by PEF. Temperature did not increase over 35°C during PEF treatment. The active site of papain, a cysteine residue, was not significantly oxidized by PEF. The breakdown of the α-helix structure was observed in PEF-treated papain by CD analysis. Inactivation of PEF-treated papain was related to the loss of α-helix structure.

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