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ORIGINAL PAPER

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SOFT WHITE CHEESE RIPENING USING BACTERIAL PROTEASE ENZYME

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ABSTRACT

Background. Microbial proteases are the most important enzymes in the industry, accounting for 60% of total enzyme sales in the world. The proteases of lactic acid bacteria (LAB) have received special attention because of their importance in the food and dairy industry.
Materials and methods. The crude extract and purified protease enzyme produced from an isolated bacterial strain identified as *Lactobacillus plantarum* were used in the ripening and flavor improvement of soft white cheese (Domiati-type). The effect of protease enzyme on the chemical and sensory properties of Domiati cheese during the storage period was studied.
Results. The results showed that the pH value, moisture and protein contents of all Domiati cheese treatments decreased by adding protease and increasing the storage period, whereas the soluble nitrogen, tyrosine and tryptophan and fat contents for all cheese treatments increased with protease addition as the storage period advanced compared to control. Moreover, most free fatty acids (FFA) contents were similar between the control and protease cheese treatments during the storage period, whereas free amino acid (FAA) content increased as the storage period for protease cheese treatments was increased. The predominated free fatty acids in Domiati cheese at the end of the ripening were palmitic acid, followed by oleic acid, stearic and myristic acids. Free amino acids (glutamic acid, proline, leucine, aspartic, lysine, serine and valine) were present in higher concentrations and represented more than 50% of total amino acids in all Domiati cheese samples at the end of the ripening period. The results of sensory evaluation indicated that as the ripening period progressed, the flavor characteristics gradually increased, leading to an improvement in the organoleptic properties compared with the control. The results showed that at end of the storage period, the cheese treatment (T2) with 2% crude enzyme recorded the highest flavor score.
Conclusion. It be concluded that the addition of 2% of crude protease enzyme accelerates the ripening process of Domiati cheese through 60 days without any defects.

Keywords: protease, Domiati cheese, ripening process, free amino acid, free fatty acid, sensory evaluation

INTRODUCTION

Proteases find huge potential in various food and feed industrial applications such as in the dairy industry for milk protein (casein and whey protein) hydrolysis while developing cheese flavor. Dairy enzymes,

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an important segment of the food enzyme industry, are used for developing and enhancing organoleptic characteristics (aroma, flavor and color) and yield of milk products. The use of enzymes (proteases, lipases, esterases, lactase, aminopeptidase, lysozyme, lactoperoxidase, transglutaminase, catalase, etc.) in the dairy market is well recognized and varies from coagulant to bio-protective enzyme to enhance the shelf life and safety of dairy products. Dairy enzymes are used for the production of cheese, yogurt and other milk products (Pai, 2003; Qureshi et al., 2015). Other proteases find applications in accelerated cheese processing and in the reduction of the allergenic properties of milk products (Qureshi et al., 2015).

The ripening of cheese is slow and very complex process. This is due to extended storage time. It involves microbiological and biochemical changes leading to the development of the flavor and texture characteristics of cheese. Several strategies have been proposed to accelerate cheese ripening. Since ripening is basically an enzymatic process, increasing the activity of key enzymes could be effected by adding commercially available enzymes to milk or curd. Several researchers examined the application of this method to accelerate the ripening of different kinds of cheeses, such as Cheddar cheese, some Egyptian cheeses, Spanish hard cheese, Tulum cheese, Mihalic cheese, Kashar cheese, and Ultrafiltered-Feta cheese (Akin et al., 2012; Karami et al., 2009; Kheadr et al., 2003; Kilcawley et al., 2012; Yilmaz et al., 2005).

Accelerating the ripening of cheese consists in accelerating proteolysis and/or lipolysis while maintaining a satisfactory texture. Proteolysis is perhaps the most important reaction during cheese ripening; it is responsible for changes in texture and flavor enhancement, since it leads to the liberation of substrates (amino acids) for the generation of sapid compounds, like amines, acids, thiols, and thioesters (Fox et al., 2000). Lipolysis is a process that releases free fatty acids, glycerol, monoacylglycerides, or diacylglycerides from triacylglycerides and is essential for the development of cheese's typical flavor (Broome et al., 2011). El-Soda et al. (1986) observed that the ripening of Domiati cheese could be accelerated, without impairing the flavor balance, using crude cell-free extracts from lactobacilli, and more particularly, *Lb. plantarum*.

Domiati cheese is the most popular soft white pickled cheese variety made from fresh cow and buffalo's milk. It is made and consumed not only in Egypt but also in the Arab world and other European countries. It closely resembles Greek Feta cheese, and it is believed that Domiati cheese originated in Egypt in 332 BC (Abou-Donia, 1986).

This study was designed to study the effect of isolated bacterial (*Lactobacillus plantarum*) protease (crude and purified enzyme) on Domiati cheese's properties during the storage period.

MATERIALS AND METHODS

Protease enzyme preparation. The protease enzyme was obtained from an isolated bacterial strain identified as *Lactobacillus plantarum* using the Biolog system (Biolog, Inc. 21124 Cabot Blvd, Hayward, CA94545 USA) in a former study (Ahmad et al., 2018) by growing the bacterial strain using sweet whey as a fermentation medium in optimal conditions. This strain showed the maximum general protease activity (8.72 U/mL) and protein content (4.13 mg/ml) with specific activity (2.11 U/mg) determined according to Beg and Gupta (2003) and Lowry et al. (1951), respectively, while the specific activity was expressed in terms of units/mg protein/ml^{-1}, according to the following equation:

$$\text{Specific activity, unit/mg} = \frac{\text{enzyme activity, unit/ml}}{\text{protein content, mg/ml}}$$

at 30°C after 48 hrs of incubation with inoculum volume 5%, fructose (5%) and yeast extract (0.5%) at pH 5.0. The bacterial culture was then centrifuged (Harrier 18/180, MSE, Huddersfield, West Yorkshire, UK) at 1400 g at 4°C for 15 min to obtain a culture supernatant (Crude enzyme). Pellets including cell debris were removed. The extracellular enzyme in the supernatant was then subjected to purification.

Purification of protease enzyme. Purification of the protease was done by standard protein purification methods which included ammonium sulphate precipitation, followed by dialysis, and Sephadex G-100 gel filtration chromatography. All purification steps were carried out at 4°C unless otherwise stated.

A-Ammonium sulphate precipitation. Protease precipitation was carried out using ammonium sulphate. A cell-free extract (supernatant) was precipitated by adding solid ammonium sulphate at saturation levels (20–60% w/v). The precipitated protein was left overnight for 24 hr at 4°C, then separated by centrifugation at 5000 rpm for 30 min at 4°C, and dissolved in 40 ml of 0.05 M phosphate buffer, pH 7.0, to obtain the concentrated enzyme solution (Abirami et al., 2011).

B-Dialysis. The resulting ammonium sulphate precipitate (enzyme suspension, 5 ml) was dialyzed against 250 ml of distilled water using acetylated cellophane tubing (12–14KD, pore size 24 A, Mediceli, International Ltd, Liverpool, London) prepared from Visking dialysis tube for 24 hr at 4°C as described by Tariq et al. (2011).

C-Size exclusion column chromatography on Sephadex G-100. The dialyzed concentrated enzyme samples of selected strains were further purified on a Sephadex G-100 column (2.5×37 cm) (Sigma Aldrich, USA). The column was equilibrated with 0.05 M phosphate buffer of pH 7.0. The dialyzed enzyme sample (3 ml) of the selected bacterial strain was loaded onto the G-100 (Sigma Aldrich, USA) column separately and then eluted with the same buffer. The eluted fractions of 5 ml volume were collected at a flow rate of 1.0 ml/min. The absorbance of each of 50 fractions was measured at 660 nm for protease activity using a spectrophotometer (Shimadzu, UV-1201, Japan). The fractions with purified protein-enzyme were stored at –20°C. Preparation of the gel column and the fractionation procedure was performed according to the method described by Sharma et al. (2006).

Domiati cheese production

Domiati cheese was made as described by Abou-Donia (2008). Fifteen liters of fresh buffalo milk (Dairy Technology Unit, Faculty of Agriculture, Cairo University, Egypt) with 6% fat concentration were pasteurized at 72°C for 15 s. The salt was added at the level of 5% during pasteurization with gentle stirring. After pasteurization, the milk was divided into five equal portions (3 liters each) and kept in five separated stainless steel containers. The milk was then cooled to 40°C. Microbial rennet powder (from *M. miehei* (Reniplus 2000 IMCU g1) was added to all portion treatments at 40°C. The first portion treatment was left free without any protease addition as a control. The crude extract at level (1 and 2%) was added to both portion treatments (T1 and T2) respectively at 40°C, and the other portion treatments (T3 and T4) were produced by adding the purified protease enzyme (4.18 unit/mg) at level 1 and 2% respectively at the same temperature. All treatments were stirred gently, and the five treatments were incubated at 38–40°C until coagulation. After that, the curd was transferred and drained through a cheese cloth placed in pierced plastic containers. After draining, the whey of each treatment was collected for pickling. The mass of Domiati cheese was cut into blocks 10–11 cm square and 7 thick, coated with polyethylene bags and packed in sterile plastic containers (capacity 250 kg). The containers were filled with the former pasteurized salted whey (7%, pH 5), and the manufactured cheese samples were stored in refrigerator at 4°C for 60 days. The chemical and sensory evaluations of cheese samples were carried out after zero, 30 and 60 days of ripening. Three replicates were carried out for each treatment and results were expressed as the mean of these.

Chemical analysis of cheese

The moisture, total fat, total protein (TP) using semi micro Kjeldahl and soluble nitrogen using TCA (12%) (SN) contents were determined according to the methods of the AOAC (2012). The pH value on warm water macerates were measured using a pH-meter (Jenway, 3510, London, UK) after calibrating the instrument with a buffer of pH 7.0.

Determination of soluble tyrosine and tryptophan contents

Soluble tyrosine and tryptophan contents of cheese samples were determined according to Vakaleris and Price (1959). Briefly, 10 ml of sodium citrate solution 0.5 M and 20 ml distilled water were added to 2.5 g of the cheese sample, mixed well using a glass rod and transferred to a volumetric flask 50 ml. The volume was adjusted up to 50 ml with distilled water. Then 2.5 ml of the HCL solution 1.41 N was added to 25 ml of the mixture and the volume adjusted up to 31.25 ml with distilled water and mixed well. Then the mixture was filtered through watt man No. 42 filter

paper. Following this, 6.5 ml of the filtrate was diluted with 6.5 ml of distilled water in a test tube and mixed well. The optical density (OD) of the above mixture was measured at two wavelengths 270 and 290 nm by the spectrophotometer (Shimadzu, UV 1201, Japan).

The soluble tyrosine and tryptophane contents were calculated from the following equations:

$$\text{Tyrosine per 100 g cheese, mg} = \\ = (0.95\ OD_{270} - 1.31\ OD_{290}) \times 0.906$$

$$\text{Tryptophan per 100 g cheese, mg} = \\ = (0.307\ OD_{290} - 0.02\ OD_{270}) \times 1.021$$

Determination of free fatty acids and free amino acids

The free fatty acids in Domiati cheese samples were estimated according to AOAC (2012) using a gas chromatography system with a FID detector (An Agilent 6890N gas chromatographic system, Agilent Technologies, Santa Clara, California, USA). The high performance amino acid analyzer (Biochrom 30, Biochrom Limited, Cambridge, UK) was used for determining free amino acids according to AOAC (2012).

Sensory evaluation

The sensory characteristics of the cheese samples stored in the refrigerator were judged by 10 untrained panelists for appearance, flavor, texture using sensory evaluation sheet, according to Pappas et al. (1996) with maximum score points of 50 points for flavor, body and texture (35 points) and 15 points for the cheese's appearance.

Statistical analysis

Statistical analysis was performed using the GLM procedure with SAS (2006) software. The analysis was carried out using the Duncan multiple ranges test to determine the differences between means of the treatments. A probability of $P < 0.05$ was used to establish the statistical significance.

RESULTS AND DISCUSSION

The effect of protease addition on chemical properties of Domiati cheese during storage period (Table 1 and Figs 1–7).

The pH values. The pH values decreased as the storage period progressed in all cheese treatments, as shown in Table 1 and Figure 1. The pH of the cheese decreased till the end of storage period. The pH values of Domiati cheese treatments made using crude and pure protease extract were lower than those of the control. This might be attributed to the greater cheese protein degradation ability by the protease extract. Moreover, even proteolytic activity may induce changes in pH values, because of the production of proteolysis compounds (e.g. sour free amino acids). Wolfschoon and Furtado (1997) mentioned that the amino acids, peptides, peptones and fatty acids forming during protein and fat breakdown result in some changes in the pH of cheese. During storage, the breakdown of proteins to nitrogen compounds by the enzymes (Fox et al., 2000) might affect the pH of cheese. Studies on pickled cheeses have reported that the pH value of cheeses changed depending on the ripening duration and temperature (Hannon et al., 2003; Hayaloglu, 2003). Statistical analysis showed that the pH of Domiati cheese treatments was highly significantly affected ($p < 0.05$) by protease enzyme addition and storage period.

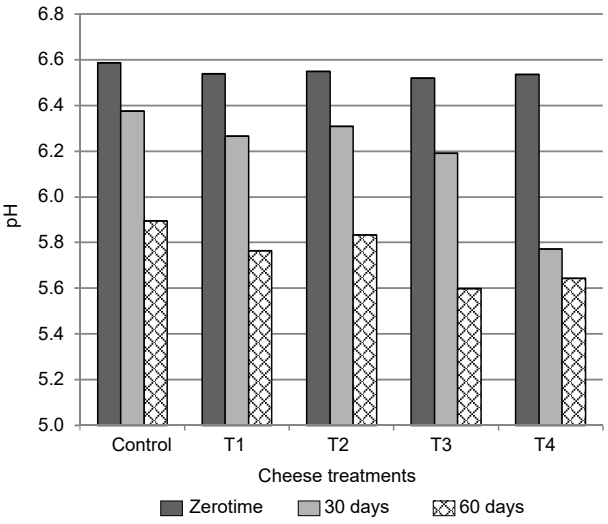

Fig. 1. Effect of protease addition on pH of Domiati cheese during storage period: Control – cheese treatment without protease, T1 – cheese treatment with 1% crude protease, T2 – cheese treatment with 2% crude protease, T3 – cheese treatment with 1% pure protease, T4 – cheese treatment with 2% pure protease

Table 1. Effect of protease addition on chemical properties of Domiati cheese

Storage period days	Treatments	Chemical properties						
		pH	moisture %	protein %	soluble nitrogen %	tyrosine mg/gm	tryptophan mg/gm	fat %
Zero	control	6.587^{a}	59.396^{a}	15.600^{a}	1.750^{g}	0.032^{h}	0.030^{h}	24^{g}
	T1	6.539^{a}	60.656^{a}	14.723^{a}	1.729^{g}	0.032^{h}	0.031^{h}	24^{g}
	T2	6.548^{a}	59.902^{a}	13.831^{a}	1.800^{g}	0.032^{h}	0.033^{h}	24^{g}
	T3	6.520^{a}	59.807^{a}	13.385^{a}	1.707^{g}	0.033^{h}	0.032^{h}	24^{g}
	T4	6.536^{a}	60.257^{a}	12.939^{a}	2.064^{g}	0.033^{h}	0.033^{h}	24^{g}
30	control	6.374^{b}	56.466^{b}	14.723^{b}	1.729^{g}	0.035^{h}	0.032^{h}	25^{fg}
	T1	6.265^{c}	57.105^{c}	13.385^{c}	1.952^{f}	0.038^{g}	0.043^{f}	27.5^{cd}
	T2	6.308^{bc}	57.367^{bc}	12.939^{d}	2.287^{c}	0.046^{f}	0.047^{de}	28^{bcd}
	T3	6.190^{d}	59.085^{a}	12.492^{bc}	2.120^{e}	0.049^{e}	0.051^{c}	27de
	T4	5.773^{f}	59.259^{a}	11.600^{f}	2.398^{bc}	0.063^{d}	0.048^{cd}	27de
60	control	5.896^{e}	55.915^{d}	13.385^{c}	2.175^{de}	0.046^{f}	0.037^{g}	26^{ef}
	T1	5.763^{f}	55.298^{ef}	12.046^{e}	2.287^{c}	0.069^{c}	0.046^{ef}	28.5^{bc}
	T2	5.833^{ef}	52.189^{g}	11.600^{f}	2.510^{b}	0.075^{b}	0.049^{cd}	30^{a}
	T3	5.599^{h}	55.454^{f}	10.707^{g}	2.398^{bc}	0.067^{c}	0.057^{b}	28^{bcd}
	T4	5.643^{g}	58.828^{a}	9.814^{h}	2.845^{a}	0.085^{a}	0.065^{a}	29^{ab}

Mean values bearing different superscripts within columns are significantly different at ($P < 0.05$) during storage period.
Control – cheese treatment without protease, T1 – cheese treatment with 1% crude protease, T2 – cheese treatment with 2% crude protease, T3 – cheese treatment with 1% pure protease, T4 – cheese treatment with 2% pure protease.

The moisture content. Figure 2 and Table 1 show that the moisture content of Domiati cheese treatments significantly decreased ($p < 0.05$) as the ripening period proceeded. This decrease could be attributed to the contraction of curd as a result of developed acidity during the pickling period, which helps to expel the whey from the cheese mass. These results are in an agreement with these reported by Kholif et al. (2010). The amino acids, peptides, peptones and fatty acids forming during protein and fat breakdown result in some changes in acidity of cheese (Wolfschoon and Furtado, 1997) and hence decrease the moisture content. Also this decrease in moisture content may be due to the osmotic pressure produced by NaCl in brine (7%) (Zerfiridis, 2001). In general, salting promotes syneresis of whey from the curd, reducing the moisture content of the cheese (McMahon et al., 2009). The moisture content of the fresh cheese treatments was higher than those during storage. The highest content of moisture was recorded with cheese made using purified protease enzyme (2%) at the end of the storage period.

Total protein content. The protein content of all Domiati cheese treatments is shown in Table 1 and Figure 3. It can be seen that the protein content of the Domiati cheese was significantly decreased ($p < 0.05$) in all cheese treatments during the 60 days ripening period. The protein content of the control cheese was higher than those made using crude or purified protease enzyme throughout the storage period. This is due to the more syneresis of whey protein from the cheese into the pickle as a result of protein degradation leading to

Fig. 2. Effect of protease addition on the moisture content of Domiati cheese during the storage period. For more details see Figure 1

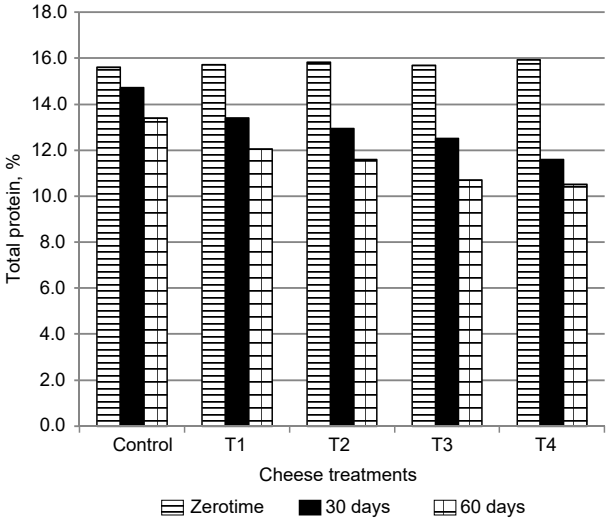

Fig. 3. Effect of protease addition on the protein content of Domiati cheese during the storage period. For more details see Figure 1

the formation of water soluble compounds and some of which were lost in the pickling solution, leading to an increase in nitrogen content in whey (Talib et al., 2009). These findings were in agreement with those reported by Nuser (2001), who found that the protein content decreased during the storage period. Decreases in protein content depending on the decrease in total nitrogen can occur due to the formation of peptides and amino acids with low molecular weights being diffused into the brine which emerges from the enzymatic proteolysis of casein (Gursoy and Kinik, 2010). In Domiati cheese, salt concentration significantly affects the protein degradation process, which in turn affects the total nitrogen of the cheese, as well as the nitrogen equilibrium between curd and whey.

The soluble nitrogen (SN) content. The effect of protease enzyme on the soluble nitrogen content of Domiati cheese treatments during the pickling period is illustrated in Table 1 and Figure 4. The cheese soluble nitrogen content of all treatments significantly increased ($P \leq 0.05$) gradually as the storage period proceeded and the protease enzyme mass ratio increased. These results were in accordance with those reported by Ezzat (1990). The results showed that cheese treatment without protease enzyme addition (control) had the lowest soluble nitrogen content. There was a positive correlation between the amount of protease enzyme and the values of ripening indices. These results could be attributed to higher proteolytic activity, which

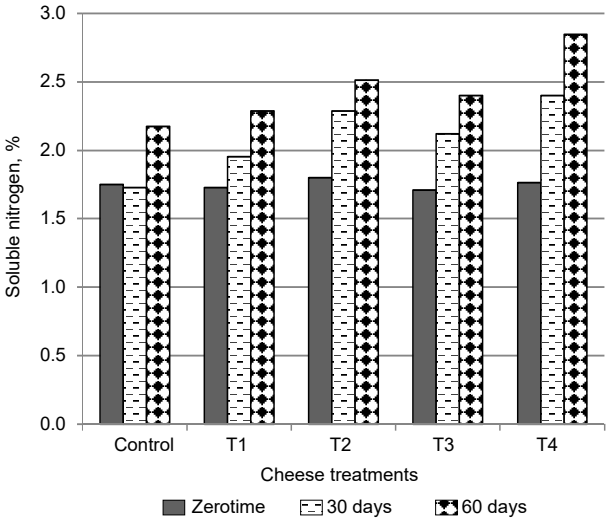

Fig. 4. Effect of protease addition on the soluble nitrogen content of Domiati cheese during the storage period. For more details see Figure 1

is important in developing the texture and flavor of cheese (Crow et al., 1994; Haandrikman et al., 1994).

Soluble tyrosine and tryptophan contents. Soluble tyrosine and tryptophan contents of Domiati cheese made using crude or purified protease enzyme during cold storage are shown in (Table 1 and Figs. 5–6) and expressed as mg per 100 g cheese. It can be noticed that the amount of soluble tyrosine and tryptophan significantly increased ($P < 0.05$) gradually with an increase in the storage period. All cheese treatments had higher values of tyrosine and tryptophan contents compared to the control. The release of soluble tyrosine and tryptophan is due to the degradation of protein by protease enzymes. The cheese made using purified protease enzyme (2%) had the highest soluble tyrosine (0.085 mg/g) and tryptophan (0.065 mg/g) contents after 60 days of the storage period. At the end of storage period, the tyrosine and tryptophan of crude cheese (2%) were 0.075 and 0.049 mg/g, respectively, while 0.046 and 0.037 mg/g were recorded for tyrosine and tryptophan of control cheese. Findings in terms of the increase in the amount of tyrosine depending on proteolysis in different cheese types are confirmed by other studies, for example Gursel et al. (2003) and Kesenkas and Akbulut (2008).

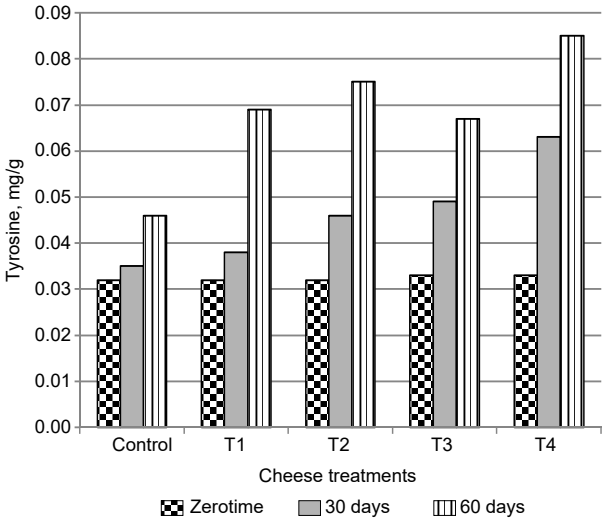

Fig. 5. Effect of protease addition on the tyrosine content of Domiati cheese during the storage period. For more details see Figure 1

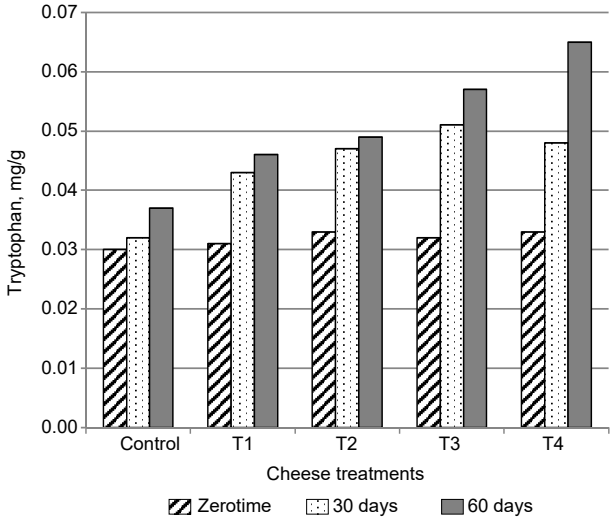

Fig. 6. Effect of protease addition on the tryptophan content of Domiati cheese during the storage period. For more details see Figure 1

Fat content. Fat content (%) in all Domiati cheese treatments as affected by adding protease enzyme is shown in Table 1 and Figure 7. The results indicated that fat content in all cheese treatments gradually increased as the pickling period proceeded. The control

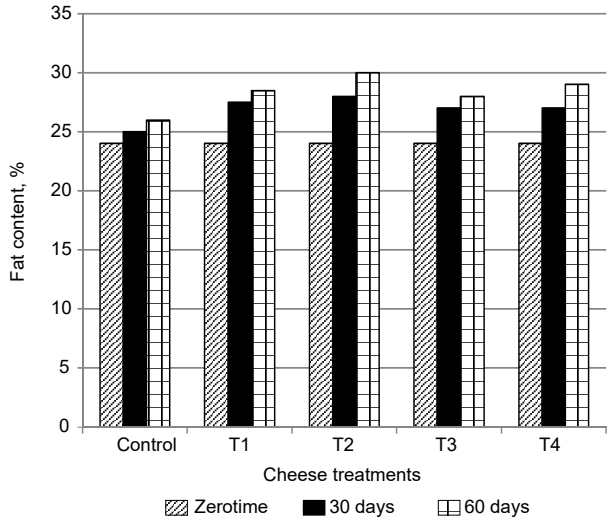

Fig. 7. Effect of protease addition on the fat content of Domiati cheese during the storage period. For more details see Figure 1

cheese sample had the lowest fat content compared to the other treatments. This is probably attributable to the decrease in solid-non-fat content as a result of protein degradation and its partial loss by solubility in whey during ripening as mentioned by Ismail et al. (2010). Moreover, an increase in fat content (% w/w) during cheese ripening in brine due to a decrease in moisture content was previously reported (Madadlou et al., 2007).

Free fatty acid analysis

Fatty acids have a direct impact on the flavor of many cheese varieties. Free fatty acids, especially short-medium chain fatty acids, are important precursors for the production of volatile flavor compounds through a series of metabolic processes during the ripening of cheese (Walstra et al., 2006). The changes in free fatty acid (FFA) contents determined by gas chromatography of Domiati cheese samples during the ripening period are given in Table 2. Free fatty acids with 4–16 carbon atoms have a considerable effect on the aroma of the cheese (Ayar and Akyuz, 2003). Among the saturated fatty acids (SFA) indicated in Table 2, like palmitic acid (C16:0), followed by stearic acid (C18:0) and myristic acid (C14:0) were most abundant. Furthermore, oleic acid (C18:1) content was the highest among the total unsaturated fatty acids (TUFA). It could be seen from the results that most free fatty acids contents were similar between control and cheese treatments made using protease enzyme (crude and purified) during the storage period.

Table 2. Changes in the free fatty acid content of Domiati cheese during the storage period, %

Free fatty acids, %	Storage periods, days														
	zero					30					60				
	treatments														
	control	T1	T2	T3	T4	control	T1	T2	T3	T4	control	T1	T2	T3	T4
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Caprlyic acid C8:0	–	–	–	–	–	0.49	0.51	0.57	0.54	0.42	0.47	0.46	0.52	0.43	0.50
Capric acid C:10	–	–	–	–	–	1.21	1.37	1.42	1.62	1.60	1.17	1.02	1.23	1.60	2.04
Lauric acid C12:0	–	–	–	–	–	1.82	1.70	1.95	1.67	1.56	1.76	1.02	1.89	1.60	1.81
Tridecanoic acid C13:0	–	–	–	–	–	0.27	0.20	0.32	0.30	0.28	0.24	0.18	0.28	0.21	0.30
Myristic acid C14:0	10.5	10.28	10.26	10.30	10.35	10.0	9.08	10.2	8.83	8.29	9.89	8.64	9.88	8.40	9.60
Tetradecenoic acid C14:1ω5	1.22	1.17	1.14	1.21	1.17	1.18	1.02	1.13	1.0	0.92	1.11	0.93	1.12	1.0	1.10
Pentadecanoic acid C15:0	2.73	2.77	2.60	2.73	2.69	2.65	2.36	2.59	2.30	2.51	2.56	2.23	2.56	2.21	2.18
Palmitic acid C16:0	32.70	32.98	32.6	33.06	32.66	32.4	29.7	32.0	28.9	31.1	32.0	31.4	31.7	27.5	29.2
Palmitolic acid C16:1ω9	2.48	2.60	2.59	2.56	2.52	2.34	2.10	2.45	2.0	2.16	2.21	2.04	2.30	2.0	1.95
Heptadecanoic acid C17:0	1.69	1.74	1.66	1.68	1.71	1.87	2.02	1.98	1.94	2.13	2.17	1.91	2.19	1.85	2.54
Hexadecatrienoic acid C16:3ω4	0.42	0.44	0.43	0.42	0.42	0.42	0.38	0.41	0.36	0.38	0.40	0.36	0.40	0.34	0.40
Stearic acid C18:0	13.38	13.53	13.03	13.38	13.41	13.25	12.0	12.9	11.6	11.7	13.0	11.56	12.7	11.0	12.5
Oleic acid C18:1ω9	23.73	24.03	24.31	23.96	23.87	24.66	27.6	25.23	28.6	29.3	26.63	30.2	27.2	30.8	26.6
Vaccinic acid C18:1ω7	4.70	4.03	4.00	3.99	3.88	–	–	–	–	–	–	–	–	–	–
Linoleic acid C18:2ω6	1.79	1.80	1.95	1.87	1.89	1.76	3.16	1.83	3.20	3.44	1.74	3.0	1.62	4.10	1.98

Table 2 – cont.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Linolenic acid C18:3ω3	0.64	0.66	0.67	0.66	0.64	0.62	2.59	0.69	2.52	4.82	0.60	0.60	0.63	1.94	0.84
Alphaoctadecatetraenoic acid C18:4ω3	1.31	1.23	1.06	1.26	1.32	1.25	1.04	1.24	1.57	1.0	1.20	1.04	1.34	1.03	1.10
Arachidic acid C20:0	0.29	–	0.38	–	0.28	0.30	0.30	0.35	0.29	0.29	0.30	0.30	0.26	0.31	0.30
Gadolic acid C20:1ω9	0.22	–	0.40	–	0.23	0.19	0.16	0.37	0.40	0.27	0.16	0.23	0.21	0.80	0.26
6-Octadecosaenoic acid C18:1ω5	–	0.74	0.76	–	0.76	–	–	–	–	–	–	–	–	–	–
Gamma linolenic acid C18:3ω6	–	–	–	–	–	0.20	0.17	0.18	0.14	0.17	0.16	0.13	–	0.14	0.16
Eicosapentaenoic acid C20:5ω3	–	–	–	–	–	–	0.11	0.13	0.13	–	–	–	–	0.14	0.14
Behenic acid C22:0	–	–	–	–	–	0.18	0.16	0.17	0.16	–	0.16	–	–	0.15	0.16
Decosenoic acid C22:1	–	–	–	–	–	–	–	–	0.37	–	–	–	–	1.20	–
C16:1ω7	0.54	0.57	0.56	0.57	0.57	–	–	–	0.30	–	–	–	–	–	–
C16:2ω7	0.36	0.39	0.36	0.37	0.39	0.35	0.32	0.37	0.30	0.28	0.34	0.30	0.38	0.30	2.54
C18:3ω4	0.33	0.30	0.33	0.35	0.34	–	–	–	–	–	–	–	–	–	–
C18:2ω4	0.68	0.65	0.62	0.68	0.67	0.65	0.60	0.65	0.60	0.60	0.63	0.57	0.63	0.60	0.62
C18:3ω6	–	–	–	–	–	–	–	0.12	–	–	–	–	0.10	–	–
C18:2ω7	–	–	–	–	0.24	–	–	–	–	–		–	–	–	–
C20:5ω3	–	–	–	–	–	–	–	–	–	–	–	0.15	–	–	–
C22:2ω6	–	–	–	–	–	–	–	–	1.16	–	–	–	–	–	–
Unidentified fatty acids	0.64	0.08	0.33	0.19	0.00	0.72	0.40	0.57	0.07	0.00	1.03	1.71	0.90	0.40	0.00

More details see Table 1.

Moreover, among the long chained fatty acids (C:16-C:20), palmitic acid (C16:0) had the highest proportion within the total fatty acid content and varied between 27.50–33.06% followed by oleic (C18:19c) and stearic acid (C18:0). Palmitoleic acid (C16:1), which is reported to indicate the level of lipolysis and to even cause defects in taste and aroma, was also found in similar amounts in control and treatments (T1, T2, T3 and T4) with protease addition. Another important fatty acid in the total fatty acid composition is linoleic acid (C18:6), which is found at higher levels in cheese treatments made using protease enzyme compared to the control cheese. As shown in Table 2, the ratios of caprlyic acid (C8:0), capric (C10:0), lauric (C12:0), and tridecanoic acid (C13:0) content of cheese samples with protease addition were higher than those of the control samples. These acids are absent in the control and all cheese treatments at zero time compared to the storage period. Similar results are reported by Türkoglu (2011) for Orgu cheese – Turkish raw ewe's cheese. He showed that palmitic, oleic, myristic and capric acids were the main free fatty acids in Orgu cheese.

It could be concluded that the ratio of fatty acids to the total amount did not change significantly during ripening.

Free amino acids analysis

The catabolism of the free amino acids during ripening produces many flavor compounds. Products of amino acid catabolism contribute as precursors to the development of volatile flavor compounds in cheese (Katechaki et al., 2009). Amino acids may contribute to flavor either directly or indirectly by serving

as precursors for volatile aroma compounds such as aldehydes, acids, alcohols, esters and sulphur compounds (Eren-Vapur and Ozcan, 2012).

Free amino acid changes in cheese treatments with protease addition and control samples during the storage period are presented in Table 3. The results showed that the free amino acid content in the control cheese without protease addition decreased during the storage period of 60 days compared to zero time. It was also found that free amino acid content of cheese treatment (T1) made using 1% crude protease increase with an increase in the storage period till 30 days then decreased, except for tyrosine, which increased until the end of the storage period. In cheese treatment T2 with 2% of crude protease enzyme, it was clear that free amino acid contents increased until the end

Table 3. Changes in free amino acid content of Domiati cheese during the storage period, %

Free amino acid	Storage periods, days														
	zero					30					60				
	treatments														
	control	T1	T2	T3	T4	control	T1	T2	T3	T4	control	T1	T2	T3	T4
Sour amino acids															
Aspartic	2.50	2.08	2.09	2.23	2.05	2.30	2.53	2.15	2.21	2.03	2.04	2.01	2.04	1.85	2.04
Glutamic	7.29	6.01	6.22	6.84	6.26	7.15	8.06	6.35	6.78	6.25	6.61	6.06	6.73	6.00	6.35
Sweet amino acids															
Threonine	1.49	1.19	1.29	1.35	1.27	1.35	1.53	1.30	1.27	1.25	1.20	1.12	1.22	1.16	1.23
Serine	1.88	1.46	1.58	1.67	1.61	1.65	1.94	1.62	1.67	1.74	1.43	1.54	1.51	1.47	1.52
Proline	3.86	3.13	3.78	4.01	3.53	3.55	4.17	3.85	3.84	3.55	3.09	3.30	3.70	2.75	3.38
Glycine	0.72	0.57	0.56	0.59	0.55	0.66	0.64	0.57	0.65	0.49	0.49	0.53	0.58	0.43	0.51
Alanine	1.18	0.93	0.98	0.97	0.89	1.02	1.21	1.02	1.15	0.89	0.93	0.85	0.96	0.82	0.95
Bitter amino acids															
Valine	1.87	1.52	1.62	1.62	1.48	1.78	1.92	1.78	1.75	1.66	1.71	1.44	1.97	1.45	1.59
Isoleucine	1.44	1.16	1.25	1.34	1.18	1.40	1.58	1.35	1.18	1.30	1.30	1.13	1.53	1.13	1.28
Leucine	3.09	2.52	2.58	2.73	2.44	2.88	2.69	2.64	2.66	2.23	2.24	2.51	2.92	1.95	2.23
Tyrosine	1.29	1.04	0.52	0.80	0.77	1.19	1.09	0.67	1.30	0.75	0.78	1.74	0.99	0.53	0.08
Phenylalanine	1.80	1.49	1.65	1.64	1.55	1.67	1.78	1.71	1.48	1.46	1.46	1.40	1.57	1.26	1.42
Histidine	1.02	0.83	0.83	0.82	0.79	0.98	0.95	0.88	0.80	0.80	0.79	0.75	0.95	0.66	0.79
Lysine	2.57	2.11	2.07	2.24	2.01	2.17	2.15	2.17	2.11	1.88	1.80	2.08	2.29	1.60	1.78
Arginine	1.15	0.97	0.98	1.08	1.02	1.05	1.18	0.99	0.98	0.95	0.96	0.89	1.03	0.80	0.95
Cystine	0.54	0.67	0.56	0.66	0.46	0.49	0.26	0.45	0.38	0.53	0.41	0.21	0.33	0.18	0.27
Methionine	0.95	0.93	1.08	0.88	0.85	0.87	0.87	0.97	0.64	0.76	0.72	0.41	0.71	0.64	0.77

More details see Table 1.

of the storage period, except for aspartic, threonine, serine, phenylalanine and proline acids, decreased after 30 days of the storage period, while cystine and methionine decreased all through the storage period. For cheese treatment T3 with 1% of purified protease enzyme, it was found that free amino acid content decreased more during the storage period than the control, except for tyrosine, valine, alanine and glycine, increased during the storage period until the 30 day point, after which it decreased. For cheese treatment T4 with 2% of purified protease enzyme, the results indicated that free amino acid content decreased during the storage period except for serine, histidine, cystine and proline decreased at 60 days. Moreover, the contents of glycine, alanine and glutamic acids were higher at 60 days compared to those after 30 days. The highest levels of most free amino acids (aspartic, therionine, serine, glutamic, alanine, isoleucine, arginine and proline) were observed with cheese treatment (T1) made using 1% crude protease enzyme after 30 days of the storage period. These results are in agreement with those reported by Azarnia et al. (2011), who observed increased levels of free amino acids in Cheddar cheeses manufactured with free exogenous amino peptidases.

Sensory analysis

The results of the sensory evaluation of Domiati cheese during the storage period are shown in Table 4. It can be seen that compared to other treatments and the control, the appearance of Domiati cheese treatments (T3&T4) made using 1 and 2% purified protease enzyme respectively decreased as the ripening period advanced. The flavor of all protease enzyme cheese treatments improved with the storage period, except T1, which decreased after 60 days because the metabolic processes during ripening are responsible for the basic flavor and texture changes (Smit et al., 2005). At end of the storage period, the cheese treatment (T2) with 2% crude enzyme recorded the highest flavor score (46.7). The results in Table 4 show that the crude protease enzyme cheese treatments (T1&T2) gained higher body and texture scores than those made with purified protease enzyme (T3&T4) during storage. Moreover, the cheese treatment (T1, 1% crude enzyme) recorded the highest body and texture score after 30 days of the storage period.

Table 4. Sensory analysis for Domiati cheese, points

Treatments	Storage period, days		
	zero	30	60
Appearance (15)			
Control	13.5	13.6	13.7
T1	13.4	13.8	13.2
T2	13.05	13.2	13.6
T3	13.5	12.9	12.5
T4	13.6	13.2	12.2
Flavor (50)			
Control	46.2	46.2	44.7
T1	45.4	48.1	41.7
T2	45.0	45.5	46.7
T3	45.7	46.4	46.5
T4	44.3	45.5	45.8
Body & Texture (35)			
Control	33.0	33.3	33.0
T1	32.4	33.8	32.8
T2	32.0	32.7	32.7
T3	32.9	32.5	31.3
T4	32.9	32.5	31.2

More details see Table 1.

The improvement in the body and texture properties of the cheeses treated through the storage period is probably due to the breakdown and hydrolysis of protein. This agrees with the findings produced by Aly and Galal (2002) and Topcu and Saldamli (2006), who reported that the textural attributes of white cheese were affected by the ripening period. Generally, the results indicated that cheese samples treated with protease enzyme extract produced a better flavor as compared to the control cheese during the ripening process. These results showed that the addition of 2% of crude protease enzyme (T2) accelerates the ripening process of Domiati cheese through 60 days without any defects in its properties.

CONCLUSION

The protease enzyme isolated from *Lactobacillus plantarum* can be used for the ripening and flavor improvement of Domiati soft white cheese. It be concluded that the addition of 2% of crude protease enzyme accelerates the ripening process of Domiati cheese through 60 days without any defects.

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