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Evaluation of enzymatic degradation of ochratoxin A by protease and lipase produced by *lactobacillus* isolated from dairy products

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Abstract

Contamination with ochratoxin A (OTA) is widespread in many foods and it is necessary to look for modern and specialized methods to remove OTA. The current study aimed to produce protease and lipase from *Levilactobacillus brevis* and *Lactobacillus plantarum*, respectively, which were isolated from Egyptian soft cheese, and evaluate them on enzymatic degradation of OTA in phosphate buffered saline (PBS) after treatment with doses ranging from 50 to 200U/ml for 6, 2 and 18h at 37 °C. The results showed that 60–80 % saturation ammonium sulfate gave the highest protease activity (1295.95 U/ml) and specific activity (3570.11), while lipase recorded high-value activity (15608.2 U/ml) and Specific activity (40021.1) at 40-60% ammonium sulfate. Partially purified using Sephadex G -100 showed only one peak for protease and lipase at fraction 55 and 32, respectively. The results indicated that using protease and lipase at 200 U/ml gives the highest degradation for OTA after incubation for 18h were 82.6 and 77.3%, respectively. Enzymatic degradation of OTA indicated that the efficacy of protease and lipase for degradation of OTA increases with the duration of incubation and the dose of the enzyme.

Keywords: Ochratoxin A (OTA); Enzymatic degradation; Protease; Lipase; *Lactobacillus strains*

1 Introduction

Ochratoxin A (OTA) is a secondary fungal metabolite produced by *Aspergillus* and *Penicillium* spp in warmer and colder climates both. OTA is a potent nephrotoxic, and hepatotoxic. It is classified by IARC as a potential human cancer class 2B [1, 2]. OTA contamination of a wide variety of food and feed as well as plant and animal products. Prevention of OTA contamination is preferable. But in fact, it is almost impossible to completely prevent its contamination in human and animal food. Therefore, implementing various measures to detoxify food and food contaminated with OTA is more feasible and necessary [3]. Consequently, there are many methods for OTA decontamination, but some of them are ineffective and produced toxic compounds and some of which cause deterioration nutritional value of food [4]. Accordingly, there is a necessary and urgent need to find an effective and safety to decontamination or degradation of OTA, So Enzymatic degradation is one of the most promising methods in this field [5]. This may be due to enzymes allowing a specific, probably irreversible, environmentally friendly and effective approach, with a minor impact on food sensory and nutritional quality [6]. So that reduction of mycotoxins using of enzymes may become a promising choice [7]. Enzymes hydrolyse the OTA amide bond, but only a few have been isolated and characterized. Two categories of carboxypeptidases A and Y (CPA and CPY) were associated with the degradation of OTA [8]. Previous studies mentioned some enzymes belonging to *Aspergillus* species are also able to perform OTA hydrolyses, such as lipases (EC 3.1), amidases (EC 3.5.), and commercial proteases (EC 3.4) [9-11]. Many enzymes produced by extracellular microorganisms may be applied to eliminate or degrade mycotoxins in contaminated food and feed to mitigate hazards to human and

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animal health [12]. So, the objective of this study is produced partially pure protease and lipase from two selected strains *Levilactobacillus brevis* and *Lactobacillus plantarum*, respectively, which isolated from Egyptian soft cheese and evaluate them on enzymatic degradation of OTA at doses ranged from 50 to 200U/ml at three incubation periods.

2 Material and methods

2.1 Chemicals

OTA chemical standard (purity > 99%) and 2, 20-azino-bis 3-ethylbenzothiazoline-6-sulphonate (ABTS) were supplied by Sigma-Aldrich (Milan, Italy), sephadex G-100 from (Aldrich Chemica Company, Inc., USA). All solvents (HPLC grade) were purchased from VWR International Srl (Milan, Italy). Water Millipore Milli-Q system (Millipore, Bedford, MA, USA) and RC 0.2 _m (regenerated cellulose membranes) filters were obtained from Grace (Deerfield, IL, USA). The SMS was supplied by the mushroom farm De Biase s.r.l. (Castellaneta, Italy). Coomassie brilliant blue G-250 dye was purchased from Bio-Rad, USA. Other chemicals were of analytical grade.

2.2 Lactic acid bacteria strains

In this study two strains *Levilactobacillus brevis* and *Lactobacillus plantarum* were used to production of protease and lipase, respectively. Which isolated from some soft cheese, these strains identified according to their morphological and cultural characteristics (Bergey's Manual of Systematic Bacteriology, 2009) and using -50CHL API- 20STREP identification system (BioMerieux) [13].

2.3 Preparation of strains

L. brevis and *L. plantarum* were propagated overnight (16 h) in 100 ml MRS, MRS modified and M17 broth (pH 6.8). A cell free solution was obtained by centrifugation of culture at 5000 rpm at 4°C for 20 min, followed by filtration of supernatant through sterilized filter (0.2 µm, Millipore), to take all cells.

2.4 Determination of protein content

Protein content was determined calorimetrically at 595 nm using Coomassie brilliant blue G-250 (CBB) and bovine serum albumin (BSA), according to Bradford (1976) protein solution containing 10 to 100 πg protein in a volume up to 0.1 ml was pipetted into 12 x 100 mm test tubes. The volume in the test tube was adjusted to 0.1 ml with appropriate buffer. Five milliliters of protein reagent were added to the test tube and the contents mixed either by inversion. The absorbance at 595 nm was measured after 2 min and before 1 h in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent. Standard curves were prepared and used in the determinations [14].

2.5 Proteolytic activity determination

Protease activity of supernatant was determined by the method of Chopra and Mathur, (1983) [15]. One ml of the substrate (1% casein in 0.05 M phosphate buffer, pH 7.0) was incubated at 37 °C for 15 min, then 1 ml of the crude enzyme supernatant which was obtained by centrifugation (8000 ×g at 4 °C for 20 min) was added. After mixing, the reaction mixture was incubated at 37 °C for 20 min. The reaction was terminated by adding 2 ml of 0.4 M trichloroacetic acid (TCA) then filtrating. The mixture was further incubated at the same temperature for 20 min. For the blank, the substrate was precipitated with TCA before adding the enzyme solution and then treated as described above. To 1 ml of the filtrate obtained after TCA precipitation, 5.0 ml of 0.4 M sodium carbonate solution was added followed by 1 ml of folins reagent and incubated at 37 °C for 20 min for color development and reading absorbance at750 nm. A unit of protease activity is defined as the amount of enzyme required to release TCA - soluble fragment giving a blue color equivalent to one µg of tyrosine under the same condition of the assy.

2.6 Lipase Activity

Enzyme activity was determined using Tween- 20 as substrate according to Von Tigerstrom and Stelmaschuk (1989) using a mixture of 2% Tween-20 in 20mM Tris- HCL buffer pH 7, 120 mMCaCl2 and 0.3 mL of enzyme source. The reaction was followed periodically by measuring the increase in optical density at 500 nm due to the hydrolytic release of the fatty acids from Tween-20 and their precipitation as the calcium salts. The reaction was carried out for a period of 30 min at 37 ◦ C. One enzyme unit was defined as the amount of enzyme that release fatty acids - calcium complex equivalent to 0.01 OD at 500 nm under standard reaction conditions [16].

2.7 Purification of Protease and Lipase enzyme

2.7.1 Precipitation by ammonium sulfate

Crude enzyme extract was precipitated by different concentrations of ammonium sulfate (20, 40, 60, 80 and 100% saturations), according to **Sethi *et al.*, (2016).** Suitable quantity of solid ammonium sulfate was added to the supernatant and then centrifuged at 8000 xg for 20 min. The precipitate was collected with minimum quantity of 0.1 M Tris- HCL buffer at pH 7.0. The supernatant fraction was dialyzed against the same buffer using dialysis bag and kept in the refrigerator for 48 h [17].

2.7.2 Gel Filtration by Using Sephadex G-100

Strains was purified by gel filtration method reported by Dioxn and Webb (1968) using Sephadex G-100, column successively (2.5 x 40 cm) (Pha-macia, Uppsala, Sweden) and eluted with 0.1 M Tris- HCL buffer pH 7.0 at a flow rate of 0.5 ml. min^{-1}. Five ml fractions were collected and assayed for enzyme activity and protein concentrate (mg/ml) at 280 and 260 nm [18].

2.8 Preparation of Phosphate buffered saline(PBS)

Phosphate buffered saline was prepared by dissolving potassium chloride (0.2 g), potassium dihydrogen phosphate (0.24 g), anhydrous disodium hydrogen phosphate (1.44 g), and sodium chloride (8 g). Start with 800mL of distilled water to dissolve all salts. Adjust the pH to 7.4 with HCl. Add distilled water to a total volume of 1 liter [19].

2.9 Degradation assay of OTA in contaminated PBS.

Protease and lipase separately were tested at five different doses (50, 100, 150 and 200U/ml) for their ability to degrade OTA in contaminated PBS. Enzyme was added to 100mL PBS contaminated with a standard working solution of OTA at 10ppb. Samples were incubated at 37ºC for 6, 12 and 18h. OTA was extracted and determination with for each dose and incubation period. All treatments were three replicates for each incubation time and enzyme.

2.10 Extraction and determination of OTA using HPLC

OTA was extracted and cleaned up using the method described in AOAC, (2007) using the IAC (OchraTest ®-p affinity column), then concentrations of OTA were determined by High- Performance Liquid Chromatography (HPLC) system [20].

2.11 Statistical analyses

All data were statistically analyzed using the General Linear Model procedure of the SPSS ver. 18 (IBM Corp, NY). The significance of the differences among treatment groups was determined by Waller–Duncan k-ratio. All statements of significance were based on the probability of (P- ≤ 0.05) was considered to be statistically significant.

3 Results and discussion

3.1 Production and Purification of enzymes extracellular

The data in (Table 1 and 2) give the summary of the purification of protease and lipase enzymes by $(NH_4)_2SO_4$ fractionation was used in this study for the first purification steps of the enzyme. The crude extract of (50 ml in 0.1 M Tris -Hcl pH 7) was tried with different ammonium sulphate concentration (0–90 % saturation). The results in (Table 1) showed that 60–80 % saturation gave the highest protease (1295.95 U/ml), Specific activity (3570.11), total activity (6479.75 U/ml), yield (37.66 %) and rate purification (2.48) accordingly; the range of 60-80 % was selected for potential purification of the protease from *L. brevis*. Concerning, lipase data in (Table 2) showed that fraction 40–60 % saturation gave the highest activity, Specific activity, total activity, yield (%) and rate of purification. The degree of saturation of ammonium sulphate greatly affected Lipase. The results showed that 40–60% saturation gave the highest lipase activity (15608.2 U/ml), specific activity (40021.1), total activity (78041.0 U/ml), yield (21.50 %) and rate purification (1.433) accordingly; the range of 40-60 % was selected for potential purification of the lipase from *L plantarum*. While, purification results of the protease and lipase using Sephadex G-100 summarized in (Table 3 and 4) recorded (6.626) rate purification with a yield (9.15 %), and (9539.69) as specific activity for protease. Moreover, lipase showed (2.99) rate purification with a yield of 3.68 % and specific activity of 83566.8. The partially purification of protease and lipase showed only one peak with protease (at fraction 55) and two peaks with protein concentration as shown in (Figure 1). On the other hand, lipase showed only one peak with Lipase (at fraction 32) and two peaks with protein concentration as shown in (Figure 2). This was obtained when the dialyzed pooled fraction was loaded on Sephadex G-100 column

equilibrated with 0.1 M Tris HCL buffer pH 7. There are many methods to purify enzymes that combination of ammonium sulfate precipitation and gel exclusion but with lower yield. As well purification by ion exchange and hydrophobic interaction chromatography. Partial purification using Sephadex G -100 increases the concentration of the enzyme so that the enzyme activity increases. Interfering some pollutants with enzymes causes enzyme instability, which reduces or eliminates its activity [21, 22]. Lipase and protease produced by microbial are more valuable compared to those derived from plants or animals due to their variety of catalytic activities available, high yield production, simplicity of genetic manipulation, absence of seasonal fluctuations, regular supply, more stability, and more safety and convenience, and the growth rate of microorganisms is very high in economical media. [23, 24].

Table 1 A preliminary ammonium sulphate saturation fractionation on protease from *L. brevis*

Ammonium Sulfate Saturation (%)	Volume (ml)	Protease activity (U/ml)	PC (mg/ml)	Total Protease	Total PC	Sp. Protease (U/mg)	Yield (%)	Rate purification
Crude enzyme	50	344.05	0.239	17202.5	11.95	1439.53	100	1.00
0-20-	5	277.65	0.681	1388.25	3.405	407.70	8.07	0.283
20-40	5	327.21	0.555	1636.05	2.775	589.56	9.51	0.409
40-60	5	1129.37	0.385	5646.85	1.925	2933.42	32.82	2.037
60-80	5	1295.95	0.363	6479.75	1.815	3570.11	37.66	2.48
80-100	5	30.761	0.0848	153.805	0.424	362.74	0.894	0.226

PC= Protein content; **Sp=Specific activity** (activity / protein content); **Total activity =**activity ×volume of fraction; **Yield =** total activity of the fraction / total activity of crud enzyme × 100; **Rate purification =** Specific activity of fraction / specific activity of the crude fraction

Table 2 A preliminary ammonium sulphate saturation fractionation on Lipase from *L. plantarum*

Ammonium Sulfate Saturation (%)	Volume (ml)	Lipase activity (U/ml)	PC (mg/ml)	Total Lipase	Total PC	Sp. Lipase (U/mg)	Yield (%)	Rate purification
Crude enzyme Homogenate	50	7258.12	0.26	362906	13.0	27915.8	100	1.00
0-20	5	1186.46	0.81	5932.30	4.05	1464.75	1.63	0.052
20-40	5	7023.53	0.53	35117.7	2.65	13251.9	9.67	0.475
40-60	5	15608.2	0.39	78041.0	1.95	40021.1	21.50	1.433
60-80	5	1596.30	0.35	7981.50	1.75	4560.85	2.199	0.163
80-100	5	163.149	0.188	815.745	0.94	867.813	0.223	0.031

PC= Protein content; **Sp=Specific activity** (activity / protein content); **Total activity =**activity ×volume of fraction; **Yield =** total activity of the fraction / total activity of crud enzyme × 100; **Rate purification =** Specific activity of fraction / specific activity of the crude fraction.

Table 3 Purification steps of protease enzyme from *L.brevis* using ammonium sulfate and gel filtration (Sephadex G-100)

Purification step	Volume (ml)	Protease activity (U/ml)	PC (mg/ml)	Total Activity	Total PC	Sp. Protease (U/mg)	Yield (%)	Rate Purification
Crude enzyme	50	344.05	0.239	17202.5	11.95	1439.53	100.0	1.00
Ammonium sulfate saturation (60-80%)	5	1295.95	0.363	6479.75	1.815	3570.11	37.66	2.48
Sephadex G -100	5	314.81	0.033	1574.05	0.165	9539.69	9.15	6.626

PC= PC=Protein content; **Sp.=Specific activity** (activity / protein content); **Total activity =**activity ×volume of fraction; **Yield =** total activity of the fraction / total activity of crud enzyme × 100; **Rate purification =** Specific activity of fraction / specific activity of the crude fraction

Table 4 Purification steps of Lipase enzyme from *L. plantarum* using ammonium sulfate and gel filtration (Sephadex G-100)

Purification step	Volume (ml)	Lipase Activity (U/ml)	PC (mg/ml)	Total Activity	Total PC	Sp. Lipase (U/mg)	Yield (%)	Rate Purification
Crude enzyme	50	7258.12	0.26	362906	13.0	27915.8	100.0	1.00
Ammonium sulfate saturation (40-60 %)	5	15608.2	0.39	78041.0	1.95	40021.1	21.50	1.433
Sephadex G -100	5	2674.14	0.032	13370.7	0.16	83566.8	3.68	2.99

PC= Protein content; **Sp.=Specific activity** (activity / protein content); **Total activity =**activity ×volume of fraction; **Yield =** total activity of the fraction / total activity of crud enzyme × 100; **Rate purification =** Specific activity of fraction / specific activity of the crude fraction

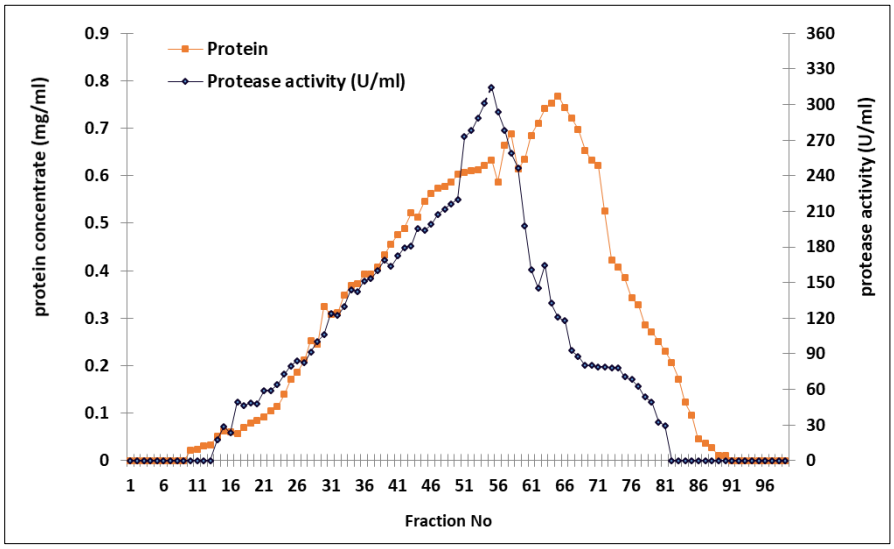

Figure 1 Gel filtration for the chromatography of protease activity on a Sephadex G-100 column

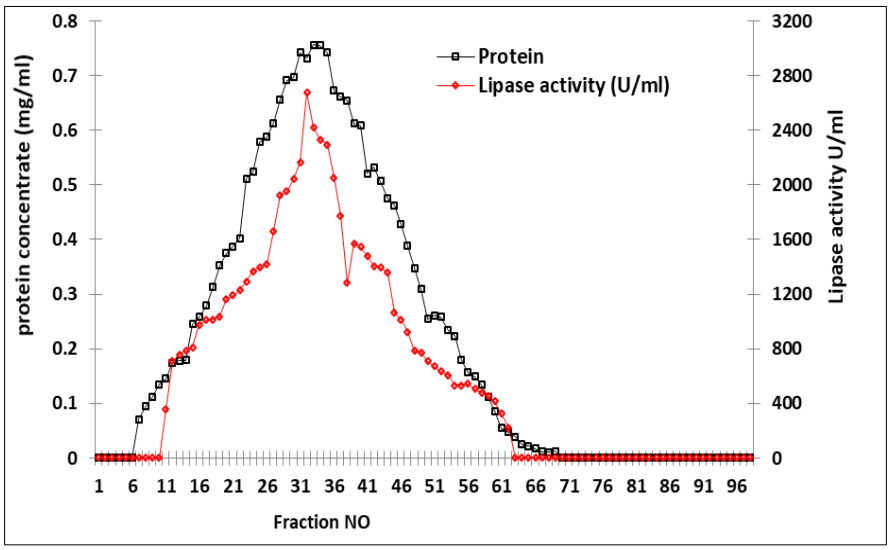

Figure 2 Gel filtration for the chromatography of lipase activity on a Sephadex G-100 column

3.2 Enzymatic degradation of OTA

Data presented in (Fig 3) showed the percentage of degradation of OTA in contaminated PBs by 50 ppb when treated with protease at 50 U/ml after an incubation period of 6, 12, and 18h were 15.1, 22.1, and 37.03%, respectively. Adding 100 and 150 U/ml from protease leads to degradation of OTA to 52.3 and 71.6% after 18h at 37°C. When using (200 U/ml) OTA degrades to 46.1, 68.5, and 82.6% at the same incubation time of 6, 12, and 18 h, respectively. The obtained data shown in (Fig 4) reflected that OTA after incubation for 18h with 50, 100, 150, and 200 U/ml from lipase was degraded to 33.9, 39.7, 64.9, and 77.3%, respectively. The ANOVA analysis in Tables (5 and 6) indicated that there are significant differences at ($p \leq 0.05$) between the incubation period and concentrations of enzyme on the degradation of OTA. OTA is hydrolyzed by enzymes through hydrolysis of the amide bond between the isocoumarin residue and phenylalanine, resulting in the formation of phenylalanine and ochratoxin α (OTα) as shown in (Fig 5) [25, 26]., which has been excreted into urine and feces. In this regard, Stander et al (2000) reported that OTA hydrolyzed by carboxypeptidase produced by bacteria and fungi to OTα [27]. In another study Berger et al (2015) found that the main degradation products of OTA were OTα, ochratoxin α amide and ochratoxin A-d5 which have MW (239.60 MW), (256.40 MW) and (408.53 MW), respectively. these products do not have a negative effect on health [28, 29]. Intracellular enzymes were capable of degrading OTA faster than its viable cells [30].

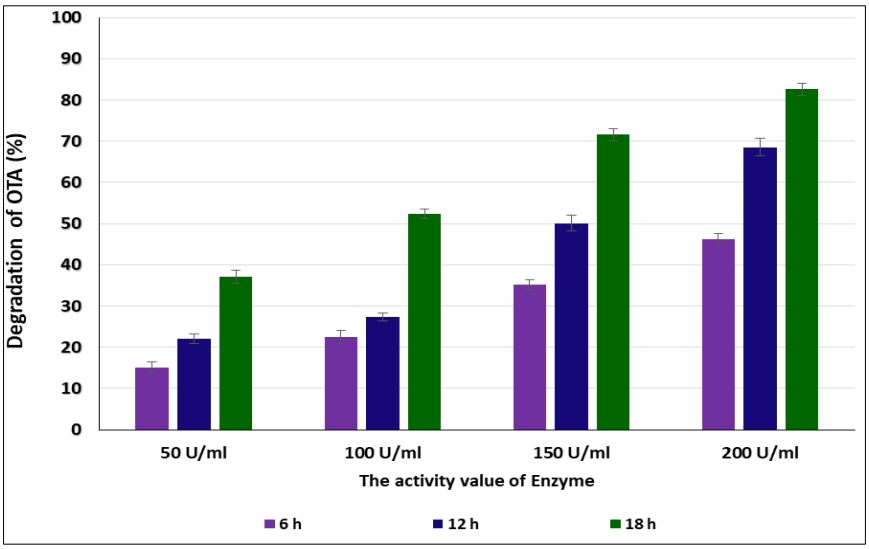

Figure 3 The percentages of reduction OTA after adding protease enzyme at 37°C with three-time incubation

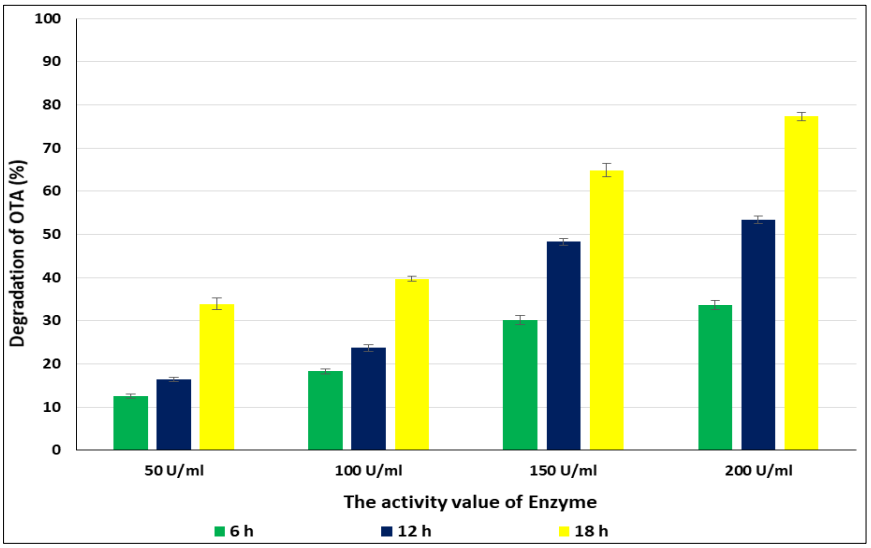

Figure 4 The percentages of reduction OTA after adding Lipase enzyme at 37°C with three-time incubation

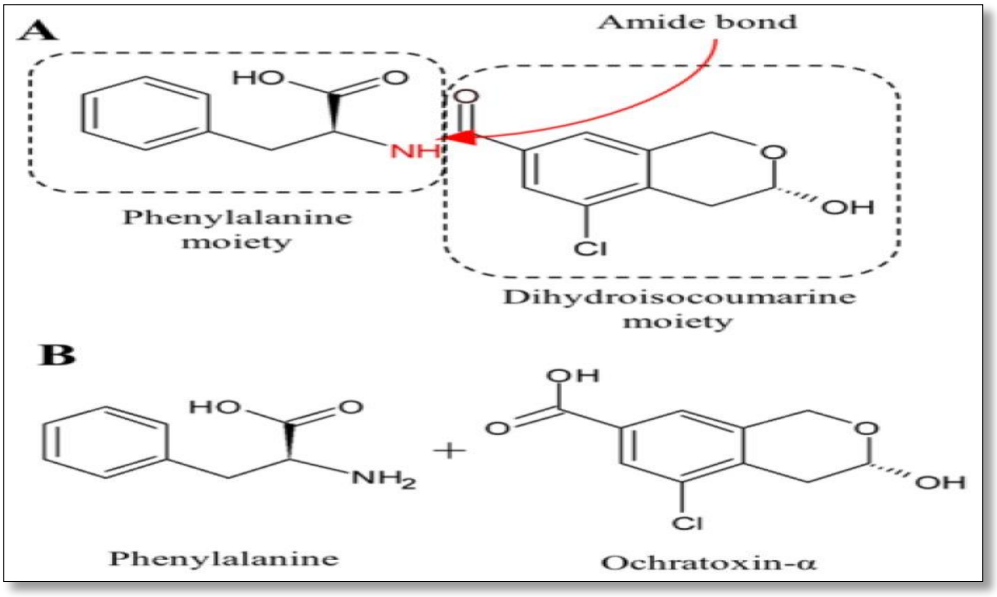

Figure 5 The structures of OTA (A) and its degradation products, ochratoxin-α and phenylalanine (B)

Table 5 ANOA for the effect of protease on content of OTA in PBs

Source	**SS**	**df**	**MS**	**F**	***P***
Intercept	60581.62	1	60581.62	8443.431	0.00000
Incubation period (T)	5195.416	2	2597.708	362.0499	0.00000
Concentrations of the enzyme (C)	6774.002	3	2258.001	314.7039	0.00000
T*C	599.8244	6	99.97074	13.9332	0.00000
Error	172.2	24	7.175	-	-
Total	73323.06	36	-	-	-

SS: Sum of Squares, df: degree of freedom, MS: mean square, P: probability at confidence 0.95.

Table 6 ANOA for the effect of lipase on content of OTA in PBs

Source	**SS**	**df**	**MS**	**F**	***P***
Intercept	51113.67	1	51113.67	23712.53	0.000000
Incubation period (T)	5613.257	2	2806.629	1302.044	0.000000
Concentrations of the enzyme (C)	7015.801	3	2338.6	1084.918	0.000000
T*C	604.645	6	100.7742	46.7509	0.000000
Error	51.73333	24	2.155556	-	-
Total	64399.11	36	-	-	-

SS: Sum of Squares, df: degree of freedom, MS: mean square, P: probability at confidence 0.95

4 Conclusion

In the current study, protease and lipase were purified using a combination of ammonium sulphate precipitation, and Sephadex G-100 chromatography, the range of 60-80 and 40-60% were selected for potential purification of both followed by using sequential chromatography technique of the most active fraction on Sephadex G 100, gave the purification to 6.6 and 2.99 folds with 9.1% and 3.68 % recovery to protease and lipase, respectively. In addition, degradation assays for OTA by both enzymes in (PBs) incubated at 37ºC for 6, 12 and 18h. The results revealed that

incubation with protease at 50 and 200 U/ml for 18h degraded the OTA to 37.03 and 82.6%, respectively. In cases lipase the OTA degraded to 33.9 and 77.3% after incubation with at the same dose and time. The results concluded that the degradation of OTA depends directly on the doses used for the enzyme and the incubation period.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest, financial or otherwise.

Availability of data and materials

The data supporting the findings of this research are available in the manuscript.

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