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Full Length Research Paper

Purification and characterization of a keratinase from the feather-degrading cultures of *Aspergillus flavipes*

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Keratinase was purified and characterized from the solid cultures of *Aspergillus flavipes* using chicken feather as substrate under solid state fermentation. The enzyme was purified by about 2.67 fold compared to the crude enzyme preparation. 40-60% ammonium sulphate saturation was used followed by anion exchange chromatography and gel filtration. The molecular weight of the enzyme under denaturing conditions was 60 KDa. The optimum pH and pH stability range were 7.0 and 5-8, respectively. The enzyme activity was significantly inhibited by incubating with EDTA, Hg^{+2}, Fe^{+3}, while it was not apparently affected by the presence of Zn^{+2}, Mg^{+2} and Cu^{+2}. The enzyme has a wide proteolytic activity towards casein, albumin and gelatin, related to the standard keratin, which makes their wide biotechnological applications justifiable.

Key words: Keratinase, *Aspergillus flavipes*, solid state fermentation.

INTRODUCTION

Keratinases (EC. 3.4.21.11) a group of serine metallo-proteases enzymes, release the free amino acids from keratinous proteins. Keratin is an insoluble, high stable protein found mostly in feathers, wool, nails and hairs of vertebrates (Shih, 1993). Keratin is resistant to the common proteolytic enzymes, papain, pepsin and trypsin (Papadopoulos et al., 1986). The high resistance of keratin to proteases may be attributed to the molecular conformation of their structural amino acids, that is tightly packed in the α-helices (hairs) and β-sheets (feather) in the presence of cystine disulfide bonds, hydrogen bonds and hydrophobic interactions (Parry and North, 1998). The fibers of keratins on both forms are intensively twisted forming micro and macro fibrils that ensure their stability against proteolysis (Krelpak et al., 2004). Feathers consist of about 5-7% of total weight of mature chicken from poultry processing plants, approximated by about million tons produced annually, worldwide. Feather from the poultry processing plant is the common source for the accumulation of more than 90% of keratinous proteins in the environment, causing pollution (Onifade, 1998). Actually, keratins of feather, nails, hairs, dead birds and animals do not accumulate in the soil, for their rapid recycling by the proteolytic enzymes (Onifade, 1998).

The extra amount of feather from poultry industries may cause a great environmental problem. The classical methods like alkali hydrolysis and pressured steam used for hydrolyzing feather may not only destroy their structural amino acids such as cysteine, serine and methionine but also require high energy (Cai, 2008; Cortezi et al., 2008). Therefore, the treatment of keratinous wastes by proteolytic enzymes is a potent technique for the nutritional and economical value of keratin feather.

The enzyme has received particular attention for its relevant applications in various types of agro and biotechnological industries. After treatment with keratinase, feather can be used as feeders, fertilizers and insoluble polymers (Yamauchi, 1996). Feather- hydrolysate can be used as food additives (Gupta and Ramnani, 2006; Williams et al., 1991). In leather and pharmaceutical products, the enzyme can be used as de-hairing agent (Thanikaivel et al., 2004). In addition to the improvement of feather meals, the enzyme can be used for production of rare amino acids such as cysteine, serine, proline and methionine (Kumar and Takagi, 1999; Riffel et al., 2007).

The enzyme production and characterization were frequently reported form various bacterial species including *Bacillus* spp. (Williams et al., 1991; Lin et al., 1992; Riffel

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et al., 2003), Actinomycetes (Ignatova et al., 1999), *Vibrio* spp. (Williams et al., 1991) and *Chryseobacterium* sp., *Burkholderia* sp. and *Pseudomonas* sp. (Riffel and Brandelli, 2002; Riffel et al., 2003; Riffel et al., 2007). Despite the high potentiality for growth and production of extracellular enzymes by filamentous fungi under solid state fermentation, compared to bacterial species, only a few studies on the production and characterization of this enzyme from fungi were documented. The fungal keratinase was only studied from *Aspergillus* sp., *Absidia* sp. and *Rhizomucor* sp. (Friedrich et al., 1999), *Aspergillus fumigatus* (Santos et al., 1996) and *A. flavus* (Kim, 2007). Unlike the physiology of bacteria, solid state fermentation is a selective environment for growth and enzymes production by filamentous fungi due to their lower water content (El-Sayed, 2009; El-Sayed, 2011).

Therefore, the objective of this study was to purify the keratinase from the solid cultures of *Aspergillus flavipes*, using chicken feather as substrate under solid state fermentation. Moreover, biochemical properties of the enzyme, compared to those from various microbial sources were investigated.

MATERIALS AND METHODS

L-Methionine, L-asparagine, L-glutamine, casein, bovine serum albumin, Nessler reagent ($HgCl_2$, KI and NaOH) and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Feathers were obtained from a local poultry breeding laboratory (Zagazig, Egypt), washed several times with hot water, milled to short fragments after drying and used as substrate for the enzyme production as described by El-Sayed (2009). All of the chemicals used were of analytical grade.

Microorganism and inoculum preparation

Aspergillus flavipes (Bain and Sart.), provided from the stock fungal cultures of the Mycology laboratory, Faculty of Science, Zagazig University, Egypt, was used for keratinase production during this study. The fungal culture was maintained on potato-dextrose agar slants, and incubated at 28 ± 1°C for 7 day. Coinidial suspension was harvested by scraping the surface of sporulating slants in 10 ml of 0.85% sterile saline solution.

Fermentation medium and growth conditions

The feathers obtained from poultry breeding laboratories were washed, air dried and their initial moisture content was measured by drying at 50°C. After cutting shortly, five grams of dried feather were dispensed into 250 ml Erlenmeyer conical flasks, then moistened with 10 ml salt solution containing 0.5% glucose, 0.25% KH_2PO_4 , 0.05% KCl and 0.05% $MgSO_4.7H_2O$ with pH 7.0 (El-Sayed, 2009). The flasks were autoclaved at 121°C for 30 min. After cooling to room temperature, the medium was inoculated with 2 ml of *A. flavipes* spore suspension, and then incubated at 28°C for 10 days, statically.

Extraction of keratinase

After incubation, the crude enzyme was extracted from feather-grown medium by the simple-buffer contact method. The solid fermented medium was mixed with 50 ml potassium phosphate buffer (pH 7.0), under shaking at 300 rpm for 30 min. The entire contents of the flasks were squeezed through a muslin cloth, and collected filtrate was centrifuged at 2683 g for 10 min to exclude remains of fungal mycelium and solid debris (El-Sayed, 2009). The clear supernatant was collected as crude enzyme preparation for further assaying.

Keratinase activity

The keratinolytic activity was determined by the method of Riffel et al. (2007) with slight modifications. Briefly, the reaction mixture contains 0.5 ml of 1.0% casein in 50 mM potassium phosphate buffer (pH 7.0) and 0.5 ml of enzyme preparation. After incubation of the reaction mixture for 1 h at 40°C, the enzymatic activity was stopped by 0.5 ml of 10% trichloroacetic acid, and centrifuged for 3 min at 5000 rpm, to exclude the precipitated protein. The released amino acids were quantified by addition of Folin-phenol reagent (Lowry et al., 1951). The developed color was measured at 650 nm, using tyrosine as standard. Blanks of enzyme and substrate were used. One unit of keratinase was defined as the amount of enzyme that liberates one µM of tyrosine per hour under optimal assay conditions. Specific activity of keratinase was expressed by the activity of enzymes per their protein content (U/ mg protein).

Protein content of keratinase

The protein content of the enzyme was determined by Folin-Phenol reagent (Lowry et al., 1951), using bovine serum albumin as a standard.

Purification of keratinase

The crude enzyme was precipitated by salting out using ammonium sulfate. The calculated amount of ammonium sulfate was added gradually to the supernatant with stirring, to obtain 40-60% saturation, incubated for 12 h at 4°C. After centrifugation at 4193 g for 15 min, the precipitated proteins were collected in a minimum volume of 50 mM potassium phosphate buffer (pH 7.0), and then dialyzed against the same buffer for 12 h. The dialysis step was repeated (three times) for the enzyme preparation under the same conditions, until there was total removal of ammonium sulfate as checked by barium chloride. The preparation was slightly concentrated by dialysis against sugar for 1 h at 4°C, followed by centrifugation at 4193 g for 5 min.

The enzyme preparation was subjected to anion-exchange chromatography using column of DEAE-Cellulose. The dialyzed supernatant was injected to DEAE-cellulose column (2 × 30 cm) which was previously washed and equilibrated with potassium phosphate buffer (pH 7.0). After equilibration, the enzyme fractions were eluted with a linear gradient of sodium chloride (20-500 mM) using the same buffer. The active fractions were pooled and collected together for subsequent purification step.

Subsequently, the active fractions from the DEAE-cellulose column were loaded to the top of Sephadex G_{200} column, and pre-equlibrated by potassium phosphate buffer (pH 7.0). The enzyme fractions were eluted by the same buffer at flow rate of 0.5 ml/min. The enzyme activity and its concentration were assayed.

Molecular weight determination

The molecular weight of the purified keratinase was determined by SDS-polyacrylamide gel electrophoresis (24). The sample was

Table 1. Purification profile of *A. flavipes* keratinase.

Purification step	Total Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude enzyme preparation	500	0.36	180	7.5	3750	20.84	100	1
Precipitation by $(NH_4)_2SO_4$ (40-60%)	250	0.31	77.5	10.4	2600	43.3	69.4	2.08
DEAE-Cellulose	28	0.20	5.6	9.0	252	45.0	6.73	2.2
Sephadex G_{200}	23	0.13	2.9	7.0	161	55.5	4.4	2.67

boiled for 5 min in a dissociation buffer containing 2% SDS and 5% 2-Mercaptoethanol. After running, the gel was stained by 0.25% Coomassie brilliant blue followed by destaining and drying (RAPIDRY-MINI). The authentic protein marker (BioLabs, New England) contains MBP-β-galactosidase (175kDa), MBP-paramyosin (80kDa), MBP-Chitin binding domain (58kDa), aldolase (46kDa), triose-phosphate isomerase (30kDa), CBD-BmFKBP13 (25kDa), lysozyme (17kDa) and aprotinin (7kDa).

Biochemical properties of Keratinase

Optimum temperature and thermal stability of enzyme

The effect of reaction temperature (20-60°C) on keratinase activity was determined by incubation of the reaction mixture for 1 h at each degree. The thermal stability of the purified enzyme was evaluated using potassium phosphate buffer (pH 7.0) at various temperature degrees (15-50°C) from 30-240 min. The residual activity was assayed by the standard method.

Optimum pH and pH stability of enzyme

The optimum pH for keratinase activity was determined using citrate-phosphate buffer (pH 3.0-6.5), potassium phosphate buffer (pH 5.8-8.0) and glycine-NaOH buffer (pH 8.0-10.6). The enzyme reaction mixture was incubated for 1h at 40°C. The enzymatic activity was assayed by the standard above method.

The pH stability of the purified keratinase was determined by preincubation of the enzyme at pH range (4.0-9.0) for 2 h, and the residual activity was measured by the normal method.

Influence of inhibitors and activators

The influence of chemical compounds on the activity of *A. flavipes* keratinase was assessed by incubation of the enzyme with metal ions (20 mM): Na^+, K^+, Fe^{+2} Mn^{2+}, Mg^{2+} Ca^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Ni^{2+}, Cu^{2+} and EDTA. The residual activity of the enzyme was measured under optimum assay conditions.

Substrate specificity

The specificity of the enzyme towards various substrates was evaluated. The ability of purified keratinase to catalyze oxidative deamination of various amino acids such as L-cysteine, L-cystine, L-tyrosine, D-valine, L-glutamine, L-glutamic acid, L-aspartic acid, and L-alanine in addition to its proteolytic activity against gelatin, albumin and casein was demonstrated. The activity of the enzyme was expressed for amino acids substrates by concentration of ammonia using direct Nesslerization method (Thompson and Morrison, 1951). The proteolytic activity of enzyme was expressed by liberation of free amino acids as detected by Folin-phenol reagent (Ledoux and Lomy, 1986). Kinetic parameters such as Km and Vmax for keratin were determined, using various concentrations of Keratin (0.2- 1.5%) as calculated from the Line weaver-Burk plot.

RESULTS AND DISCUSSION

Keratinase purification

Crude enzyme preparation was harvested from the feather-grown *Aspergillus flavipes*, under solid state fermentation. The crude enzyme was precipitated by ammonium sulfate at 40-60% saturation. The collected precipitate was subjected to dialysis against the same buffer to exclude the salt. The specific enzyme activity was increased by 2.06 with 69.4% yield by ammonium sulfate (Table 1). The dialysate was applied to the DEAE-column, and pre-equlibrated with potassium phosphate buffer (pH 7.0). Using gradient concentrations NaCl (20-500 mM) for enzyme elution, the highest keratinase active fractions (45.0 U/mg protein) were collected in 40mM NaCl. The activity of keratinase was increased by about 2.2 fold, compared to crude enzyme preparation, with 6.73% recovery. Substantially, the active fractions of DEAE-Column were combined and loaded to the top of Sephadex G_{200} column, which was previously equilibrated by the same buffer. The enzyme fractions were eluted from the gel-filtration chromatography column by potassium phosphate buffer (pH 7.0) (1/6 v/v). A single peaked fraction of keratinase was collected from the column, with specific activity of 55.5 U/mg protein, by about 2.67 purification fold over the crude enzyme preparation (Table 1). Similar purification protocols were used for keratinase from the solid cultures of bacterial species such as *Bacillus lichniformis* (Lin et al., 1992), *Chrysobacterium* sp. Kr6 (Riffel et al., 2003, 2007) and *Strptomyces* sp. (Bockle et al., 1995). However, a relativefew studies on the purification and characterization of keratinase from solid fungal cultures were documented, in spite of the higher potentiality of fungi to grow on low water content-solid substrates than bacteria, physiologically. Keratinase was frequently purified and characterized from the solid cultures of *Aspergillus*

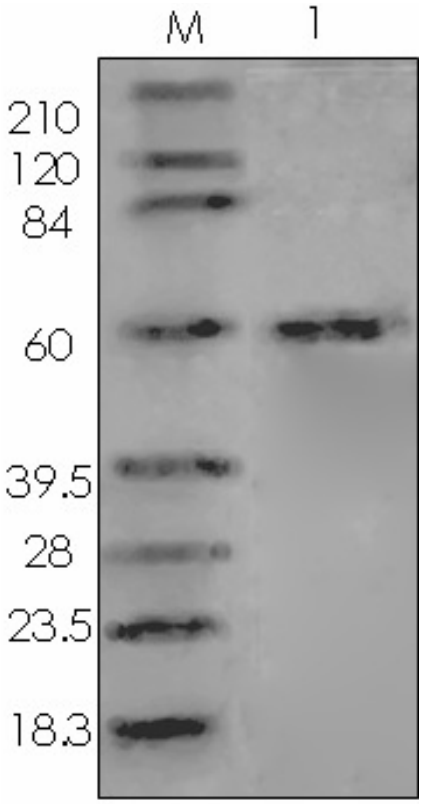

Figure 1. SDS-PAGE of the purified keratinase from *A. flavipes*. M; protein marker, S; purified keratinase.

fumigatus (Santos et al., 1996) and *A. oryzae* (Bressollier et al., 1999). Practically, the presence of multiple proteolytic and keratinolytic enzymes in feather-grown microbial cultures were reported for *Streptomyces albidoflavus* and *Kytococcus sedentarius* (Bressollier et al., 1999; Longshaw et al., 2002). Also, the cultures of *Microsporum gypsum*, *Trichophyton rubrum*, *Chrysobacterium* sp. and *Aspergillus flavipes* (Kunert, 1992; Saha et al., 2009; Riffel et al., 2007; El-Sayed, 2009) grown on feather were found to contain disulfide reductases, L-methioninases in addition to proteases and keratinases. Consequently, applying successive chromatographic techniques for purification of keratinase from the solid microbial cultures is justifiable.

Molecular weight of keratinase

The molecular weight of the purified keratinase was determined under denaturing conditions using 10% SDS-PAGE. In Figure 1, the molecular weight of the enzyme was about 60 KDa. The molecular mass of the purified keratinase from *A. flavipes* coincides with that reported for the enzyme from other microorganisms (Allpress et al., 2002; Farrag and Hassan, 2004; Kim, 2007; Riffel et al., 2007).

Biochemical properties of Keratinase

The optimum pH for the purified keratinase activity was 7.0 in potassium phosphate buffer using keratin as standard substrate (Figure 2). Practically, a gradual increase on the enzyme activity from pH 3.0 to its highest value at pH 7.0 and followed by a decrease of its lowest value at pH 10.0 was observed. At pH 3.0 and 10.0, the enzyme retains only 41.1 and 51.0% from its initial activity. Regarding the optimum pH of keratinase, the enzyme activity was significantly reduced at higher acidic and alkaline pH, which may be attributed to the enzyme denaturation/ changing on its catalytic folding state. The neutral to slightly alkaline activity of keratinases was frequently documented for this enzyme from various microbial sources (Bockle et al., 1995; Santos et al., 1996; Brandelli and Riffel, 2005).

The pH stability of purified *A. flavipes* Keratinase was evaluated by preincubation of the enzyme without substrates at different pHs (4.0-9.0). From the pH stability curve (Figure 3), it could be observed that the enzyme has maximum stability at pH 7.0 within the pH range of 5-8 for 2 h. Unlike the relative stability of the catalytic state of the enzyme in the neutral conditions, the rate of enzyme denaturation was lower in acidic conditions compared to the alkaline pHs. Practically, the enzyme lost about 16.6% at pH 5.0 compared to 26% at pH 9.0 by incubating for 2 h. This reveals the significant negative effect on the enzyme tertiary structure by the lower concentrations of H^+ ions. The pH stability of *A. flavipes* keratinase coincided with that reported by Brandelli and Riffel (2005), Riffel et al. (2007) and Tapia and Simões (2008) on bacterial enzyme, suggesting the similarity on the structural amino acids. The wide pH range of *A. flavipes* keratinase may enhance their biotechnological applications especially in leather industry and bio-remediation process, consistent with other proteases (Auld, 1995).

The impact of various metal ions and EDTA on the keratinolytic activity of *A. flavipes* keratinase is summarized in Table 2. The enzyme activity was significantly reduced by the presence of EDTA, and Hg^{+2} retaining only about 47.5 and 51.2% respectively. The higher negative effect of EDTA as metals chelating agents on the activity of keratinase ensures the metallo-proteinic nature of this enzyme. Similar results approving the metallo-dependence nature of microbial proteases were reported by various researchers (Auld, 1995; Tapia and Simões, 2008; Riffel et al., 2007). The enzyme has maximum activity by preincubating with Fe^{+3} and Co^{+2}, approving their positive effect on stabilizing the enzyme tertiary structure and catalytic state. That is consistent with those reported of L-methioninase. The negative effect of Hg^{+2} on keratinase activity may be attributed to the reaction with the metals of the enzymes or disruption of the folding state by replacing the sulfur of disulfide bonds, causing enzyme denaturation. *A. flavipes*

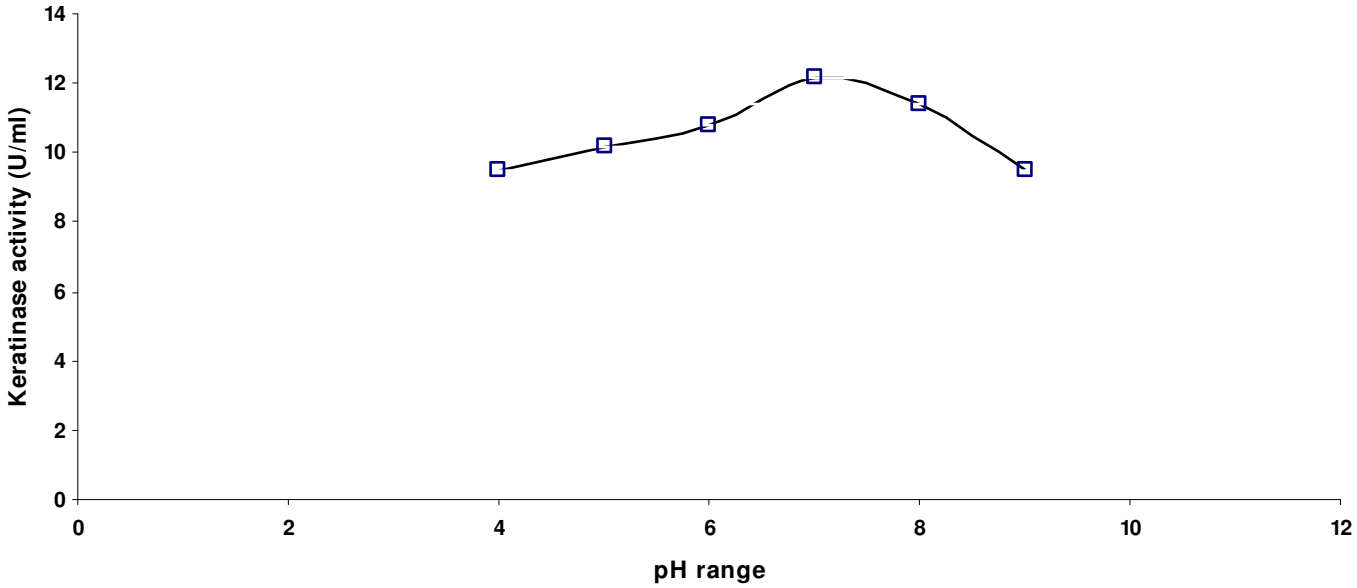

Figure 2. Effect of pH values on the activity of the *A. flavipes* keratinase.

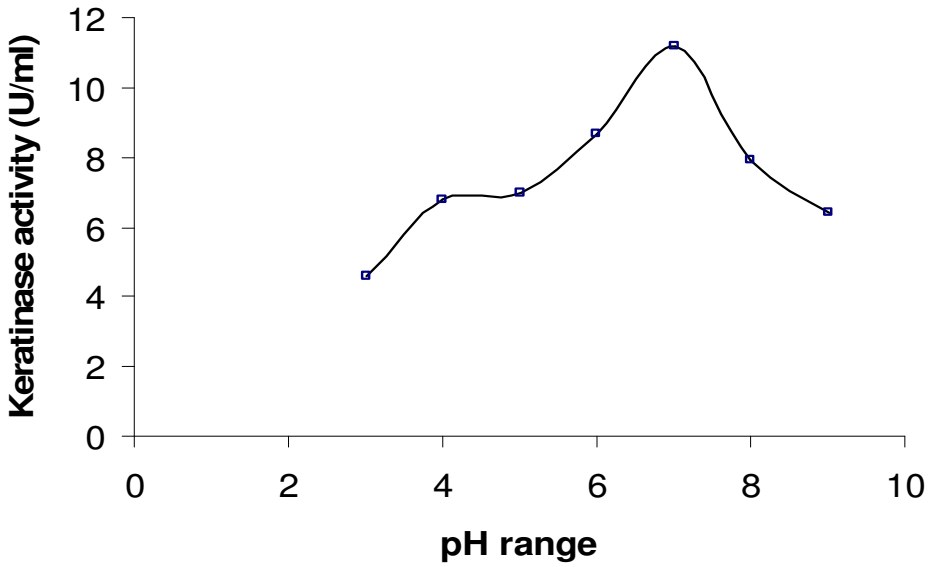

Figure 3. pH stability of the purified *A. flavipes* keratinase.

keratinase activity is relatively not affected by the presence of Zn^{+2}, Mg^{+2} and Cu^{+2}. However, the activity of enzyme is slightly stimulated by incubation of the enzyme with Ca^{+2} and Mn^{+2}, suggesting the stabilizing effect of these elements on the catalytic state of enzyme (Sangali and Brandelli, 2000). Similarly, Ca^{+2} ions were documented to be involved as stabilizing agent for the tertiary structure of microbial proteases against auto-proteolysis (Secades and Guijarro, 2001). Also, Mn^{+2} and Mg^{+2} were reported to be protecting agents against thermal denaturation by most of microbial metallo-proteases (Kumar and Takagi, 1999). Otherwise, a slightly inhibitory effect was exerted by monovalent ions as Na^{+} and K^{+} on the activity of *A. flavipes* keratinase.

Kinetically, the affinity of the purified enzyme towards various proteinous substrates was evaluated as seen in Table 3. The enzyme has a maximum proteolytic activity against keratin (100%), casein (95.1%) and albumin (80.5%) and gelatin (63.5%). The multiple proteolyic and keratinolytic activity of keratinase produced from the solid microbial cultures using chicken feather as solid substrate was frequently reported (Lin et al., 1992; Bockle et al., 1995; Santos et al., 1996; El-Sayed, 2011; Riffel et al., 2007). Also, the purified proteases from *Chrysobacterium* sp. have a highly kerationlytic/ de-hairing activity (Riffel et al., 2003). In addition, the purified enzyme has a

Table 2. Effect of some metal ions and EDTA on the activity of *A. flavipes* keratinase.

Metal ions	Activity (U/ml)	Relative activity (%)
Without	13.5	100
Na^{+}	9.1	67.5
K^{+}	11.2	78.3
Ni^{+}	16.8	124.4
Ca^{2+}	15.2	112.5
Co^{2+}	20.5	151.2
Mn^{2+}	16.8	124.4
Hg^{2+}	12.3	79.1
Fe^{3+}	20.2	150.1
Zn^{2+}	12.7	79.4
Mg^{2+}	11.2	78.3
Cu^{2+}	11.1	78.2
EDTA	12.8	79.4

Table 3. Substrate specificity of *A. flavipes* keratinase.

Substrate	Activity (U/ml)	Relative activity (%)
DL-Valine	0.54	2
L-Alanine	---	--
Cysteine	0.08	0.4
Glutamine	0.03	0.1
Glutamic acid	---	
L-Aspartic acid	0.09	0.45
L-Cystine	0.83	4.05
Gelatin	13	63.4
Albumin	16.5	80.5
Casein	19.5	95.1
Keratin	20.5	100

desulfurase activity for L-cystine and deaminating activity for D-valine and aspartic acid.

In conclusion, this study reveals the potentiality of the local isolate *A. flavipes* to degrade keratinous substrates, particularly chicken feather releasing free amino acids, through extracellular keratinolytic system. This fungal isolate was reported as non toxic strain (El-Sayed, 2011) justifying its usage in our study. *A. flavipes* keratinase was purified using different chromatographic techniques and characterized to assess its catalytic and structural identity, compared to other microbial keratinases.

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