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Purification and Characterization of a Thermostable Serine Alkaline Protease from *Bacillus stearothermophilus* AEAL2

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ABSTRACT

Local isolate *B. stearothermophilus* AEAL2 was grown under the optimum conditions in production media for 48 hrs at 55°C to produce *serine alkaline protease.*The enzyme was purified to homogeneity by employing ammonium sulfate precipitation,DEAE-Cellulose ion exchange chromatography, and Sepharose-6B gel filtration chromatography. The purification steps of protease resulted in the production of two protease fractions namely protease I (serine alkaline)and II, the obtained, purification folds were 10.65 and 69.56, and recovery were 18.5% and 22.5% respectively. The purified serine alkaline protease was completely inhibited by 10mM of phenylmethylsulfonyl fluoride (PMSF). This is in addition to that, retained 71.5% from its activity in presence of 10Mm EDTA.The purified enzyme showed extreme stability towards various surfactants such as, Tween-80, Triton X-100, SDS, and CTAB, thus advocating its suitability for various industrial applications.

INTRODUCTION

Serine alkaline proteases (SAP) are one of the most important groups of industrial enzymes. They account for approximately 35% of the total microbial enzyme sales[1]. The serine proteases are divided into twenty families and six clans according to the similarities and differences in their three-dimensional structures, amino acid sequences and active site configuration [2]. Alkaline proteases useful for detergent applications were mostly active in the pH range 8 - 12 and at temperatures between 50°C - 70°C [3]. The optimum pH range of alkaline proteases is generally between pH 9 and 11, with a few exceptions exhibiting higher pH optima, up to a pH range 12 – 13. The optimum temperature of alkaline proteases ranges from 50°C to 70°C[4]. In general, the molecular mass of protease is ranged between 15 and 45 kDa , 33.5 kDa for the *B. stearothermophilus* F1[5]. Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), diisopropylfluorophosphate (DIFP), phenylmethylsulfonyl fluoride (PMSF), and tosyl-L-lysine chloromethyl ketone (TLCK)[6]. The subtilisins (E. C. 3.4.21.14), which were defined by their catalytic mechanism as serine proteases, were amongst the first enzymes to be manufactured using recombinant strains and established themselves as benchmarks in several applications[7]. Of all proteases, alkaline proteases produced by *Bacillus* species are of great importance in the detergent industry due to their high thermostability and pH stability [8].

The strategies adopted for the purification of alkaline protease clearly notice that ammonium sulfate remains a popular agent for the concentration of protease from microbes [9]. For more enzyme purification a combination of one or more chromatographic techniques are applied via Affinity chromatography (AC) with α- casein Agarose resin was used for the purification of alkaline protease from *B. licheniformis* RSP-09-37 [10]. Ion exchange chromatography (IEC) by DEAE-cellulose resin [11], hydrophobic interaction chromatography (HIC) with Phenyl sepharose[12]and gel filtration chromatography and Sephacryl S-100 HR liquid chromatography[13].The aim of this study is to purified and characterization of a Thermostable Protease from *Bacillus stearothermophilus* AEAL2.

MATERIALS AND METHODS

The thermostable alkaline protease producing isolate of *B. stearothermophilus* AEAL2 strain was obtained from the Biotechnology Laboratory of University of Baghdad, Genetic Engineering and Biotechnology Institute for Postgraduate Studies/ Baghdad[14]. This strain has been isolated from Baghdad/ Iraqi soil and their spores were stored in soil stock at room temperature for nearly 10 years. For activation of protease producing thermophilic *B. stearothermophilus* AEAL2 isolate, 1gm of soil stock was suspended in 9ml Nutrient broth (NB), agitated vigorously in a shaker water bath at 80°C for 1hrs. Serial dilutions of the sample using sterilized normal saline were set up, then 0.1 ml aliquot of 10^{-4} and 10^{-5} dilutions was spread on skim milk agar plates and incubated at 55°C for 24hrs.

Determination of protease activity

Protease activity was measured according to the method of Brock *et al.*[15]using casein as a substrate. The absorbance was measured at 280 nm since one unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 280 nm equal to 0.01 in one minute under experimental conditions according to the following equation:

$$\textbf{Enzyme activity (U/ml)} = \frac{\text{Absorbance at 280 nm}}{0.01\times10\times0.1}$$

Protein concentration was measured according to the Bradford [16] method.

Ammonium sulfate precipitation was achieved by adding ammonium sulfate to the crude enzyme gradually with continuous mixing on the ice at saturation ratio of 75%; the mixture was centrifuged at 6000 rpm for 20 min at 4°C. The resultant pellet was dissolved in 40 ml of 0.1M phosphate buffer pH8.

Ion exchange chromatography (DEAE-Cellulose) It was prepared according to Schutte *et al.* [17]. A column (2.0x 15 cm), was washed with an equilibration buffer 0.05M Tris-base, 2µM $CaCl_2$, pH8.And the enzyme was eluted with an elution buffer and gradient NaCl in a concentration of (0 - 0.1 M).

Gel filtration was done by using Sepharose-6B gel was prepared according to the instructions of the manufacturing company (Pharmacia). It was poured with care to avoid bubbles onto a column with dimensions of (1.5x 85) cm. the column was washing by using 0.2M Tris-hydrochloride, 2µM $CaCl_2$, pH 8 buffer and flow rate was organized to be 30 ml / hrs., and a fraction volume of 5 ml was collected.

Characterization of alkaline Protease

There were some metal ion and inhibitor effects on protease activity was used as follow ethylene diamine tetraacetic acid (EDTA), Iodoacetic acid (IAA), CaCl2, and MgCl2 was prepared singly by dissolving in D.W. as stock solution to give a final concentration of 2M, then 10 mM was prepared from each a stock with D.W., while (phenylmethylsulphonyl fluoride) PMSF was prepared by dissolving in isopropanol as a stock solution to give a final concentration of 2M; then 10mM was prepared from this stock solution with isopropanol. The purified protease was pretreated for 30min at 25^{O}C with 10 mM of the following reagents (1:1v/v) before assay: IAA, EDTA, PMSF, $CaCl_2$, and $MgCl_2$. The enzymatic activity was measured under standard assay conditions.

Effect of Surfactants on Enzyme Activity

The purified protease was pretreated for 30min at a 45^{o}C with 1% (w/v) in D.W. of the following reagents (1:1v/v) before assay: (sodium dodecyl sulfate (SDS), Triton X-100, Tween-80, and CTAB). The enzymatic activity was measured under standard assay conditions.

RESULTS AND DISCUSSION

The purification of alkaline proteases is important from the perspective of developing a better understanding of the functioning of the enzyme[18]. Purification of protease from *B. stearothermophilus* AEAL2 was achieved by several steps as follows:

Firstly, the crude extracellular enzyme was subjected to ammonium sulfate precipitation with a 50-75% saturation ratio. It was found that this ratio gave a specific activity of 4318.695 Unit/mg protein. This result indicated that there was an increase in the specific activity compared with that of the crude extract (1082.02 Unit/mg protein).

Salting out using ammonium sulfate is one of the classical methods in protein biochemistry. Formerly, it was widely used for the fractionation of proteins; it is rather used as an inexpensive way of concentrating a protein extract[19]. Produced protease by *Bacillus amyloliquefaciens*35s was able to purify it first by 70% ammonium sulfate precipitation, increasing specific activity to 5143 u/mg, by 2.6 fold of that of the crude enzyme (1408.5 u/ml)[20]. Euo-Sun and Jong [21]reported that fractions of 60-80% ammonium sulfate saturation contained high protease activity and the specific activity increased from 12 Uint/mg to 38 Uint/mg protein with 3.1 purification fold as an initial step for the purification of caseinolytic extracellular protease from *B. amyloliquefaciens*[22], however, found that *B. subtilis* supernatant fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation gave an increase in the specific activity from 10 to 12 U/g protein with 1.2 purification fold; while 60-70% ammonium sulfate saturation level increased the protease activity 6.41 fold with 62% recovery, showing specific activity of 2106.02 Uint/mg for purification of alkaline protease of mutant *B. licheniformis* UV-9[23].

Later, the concentrated enzyme was successively subjected to Ion Exchange Chromatography (IEC) on the DEAE-cellulose column. The elution profile (Figure1) revealed one peak between 17 and 34. The Maximum protease activity was detected in the fractions 18-29. The active fractions were pooled and used for further study. The Ion Exchange Chromatography with DEAE cellulose enhanced the specific activity of the enzyme to 14437.5 Unit/mg protein, which is 13.34 folds. Alkaline protease from many sources purified by employing DEAE-cellulose resin such as protease from *Bacillus pumilus* is 13.2 fold of activity [24].

Finally, the enzyme collected after (IEC) was applied to a Sepharose 6B column. The results illustrated in (Figure 2) showed that there were two distinct and separated peaks which represented protease with maximum activity. Peak I protease was eluted between 21 and 28 fractions and contained 18.63% of the total protease activity. Peak II protease eluting between the 29 and 36 fractions constituted 22.51% of the original activity with an overall 69.56 folds purification. The protease enzyme from *Bacillus megaterium* was successively purified on Sephadex G-200 resulting in the production of two protease fractions, namely protease P1 and P2 with specific activities of 561.27 and 317.23 Unit/ mg of protein, respectively [25]; while, three novel thermostable proteases, designated S, N, and B, were purified from the culture supernatant

of *B. stearothermophilus* strain TLS33 by lysine affinity chromatography[26]. The results of the purification procedure are summarized in (Table 1).

Table (3.2): Summary of the purification of *B. stearothermophilus* AEAL2 protease

Purification steps	Volume (ml)	Protease Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification fold	Yields (%)
Crude extract	100	331.1	0.31	1082.02	33110	1	100
Ammonium sulfate (50-75%)	20	993.3	0.23	4318.695	19866	3.991	60
DEAE Cellulose	60	231.27	0.016	14437.5	13876.36	13.34	41.90
Gel Filtration Chromatography	25	246.80	0.47	1152.14	6170	10.65	18.63
	25	298.16	0.103	75263.69	7454	69.56	22.51

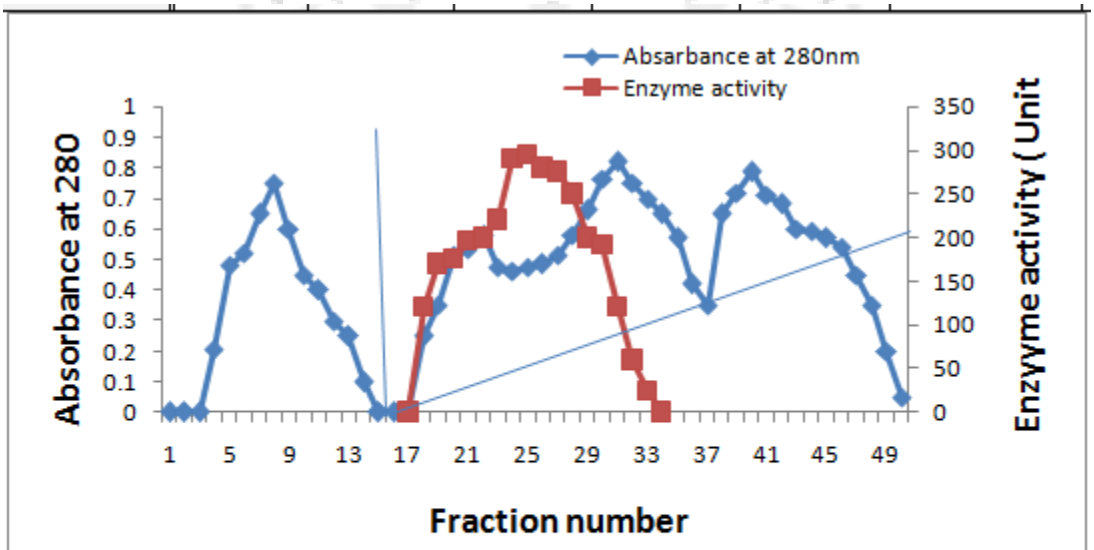

Figure (1): Ion-exchange chromatography for Alkine Protease enzyme through DEAE-Cellulose column (25 x2.5) cm. The column was calibrated with Tris-base buffer pH8.0, flow rate 60 ml /hrs and 5ml/

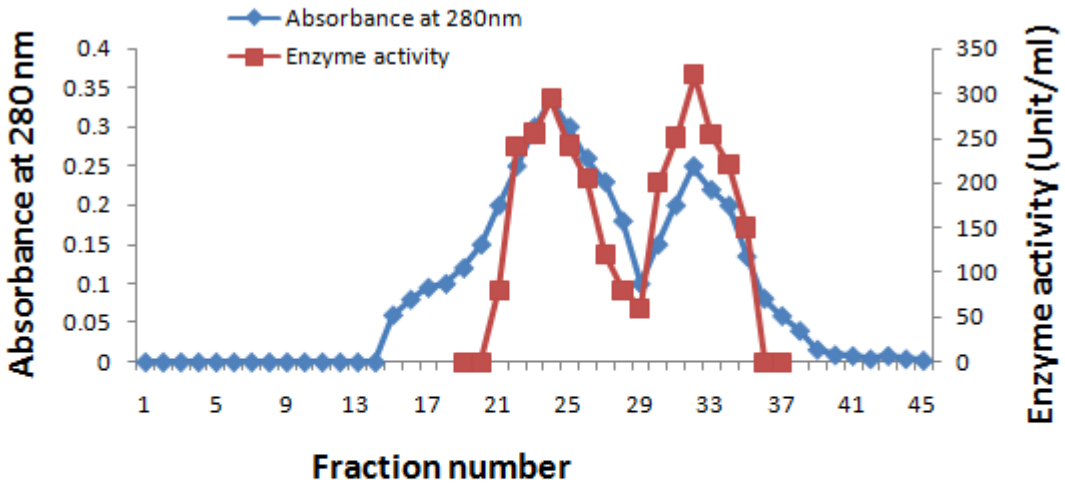

Figure (2): Gel-filtration chromatography for purification Alkaine protease by using Sepharose 6B column (1.5 x 85) cm. The column was calibrated with Tris-base pH 8.0, flow rate 50 ml/hrs and 5ml/fraction.

The effect of some metal ions ($CaCl_2$, and $MgCl_2$) on the activity of the purified proteases was tested. The results showed that the tested metal ions Ca^{+2} led to an increase in the activity (145% and 201%) of both enzymes (protease I and II) respectively, also for Mg^{+2} (60% and 180%) for protease I and II respectively (Figure 3). Confirming that these cations take part in the stabilization of the protease structure and are required for protection against thermal denaturation[27].An alkaline protease produced from a *Bacillus* sp. was stimulated by the metal ions $Ca^{2\div}$ with Mg^{2+} and Mn^{2+} with Ca^{2+} having the maximum effect. The thermal stability of the enzyme was also enhanced to varying degrees in the presence of these ions [27].

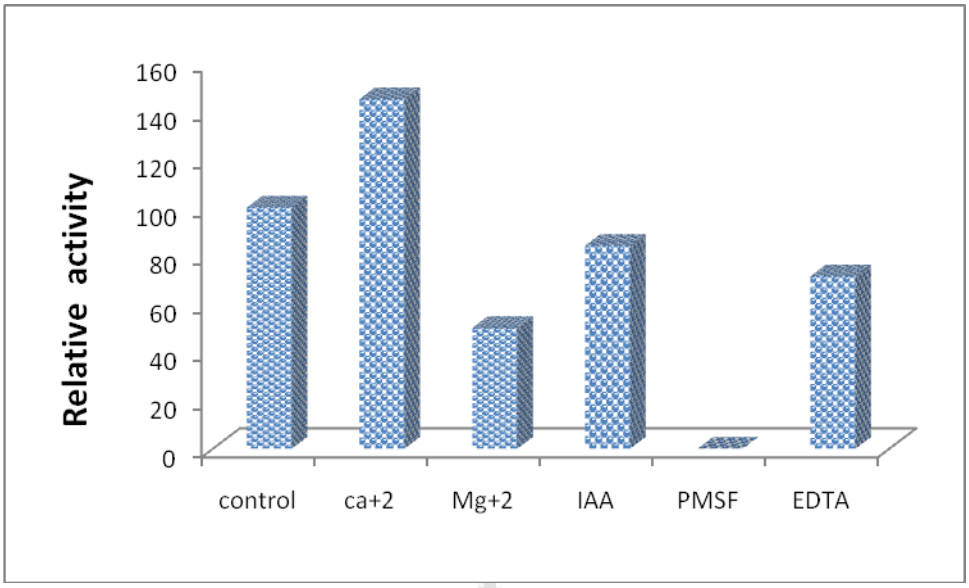

Figure (3): The effect of metal ions and inhibitors on protease activity produced by *B. stearothermophilus*AEAL2

The presence of Ca^{2+} in the medium may explain the protease stability at such temperatures as shown by Rahman *et al*. [28], they reported that the presence of Ca^{2+} 2 mM and 5 mM respectively in the reaction mixture contributed to stabilizing the protease produced by *Bacillus stearothermophilus* F1 and *Bacillus mojavensis* A21 respectively.

The effects of some protease inhibitors (Iodoacetic acid (IAA), EDTA, and PMSF) were tested to determine the type of the purified enzymes. Only phenylmethanesulfonyl (PMSF) at 10 mM was able to inhibit the protease I completely, while the enzyme retained approximately 90% of its activities in the presence of IAA. Moreover, total protease activity was retained by up to 100 % in the presence of 10mM EDTA, thereby indicating that the protease I belonged to the serine protease family. In this regard, PMSF caused sulphonation of the serine residues residing in the active site of the protease and had been reported to result in the complete loss of enzyme activity [29]. The Alkaline protease from a mutant of *Bacillus licheniformis*Bl8 was strongly inhibited by PMSF and slightly by EDTA.[30]

By contrast, protease II was inhibited up to 94% by EDTA with 10 mM concentration, (which is a metal chelating agent), while PMSF and IAA did not drastically affect the activity of the enzyme (Figure 3). This clearly indicated that protease II was a member of the metalloprotease

family. In previous studies [31-32] and Wu *et al*.[33] also found that Ca^{2}+ activated and EDTA inhibited the protease activity. These findings were in agreement with the findings of Siriporn *et al*. [34], where 94% of the protease produced from *B. subtilis*and*B. megaterium*, respectively was inhibited by the EDTA. Sharmin and Rahman[35] also reported that the protease produced from *Bacillus* strain FS-1 was inhibited by the EDTA. The activity of protease purified from *Xenorhabdus nematophila* was totally abolished by 1 mM EDTA, but not affected by cysteine, serine and aspartyl protease inhibitors [36].The *B. stearotbermopbilus* RM-67 protease I was inhibited by metal chelating agents 8-hydroxyquinoline-5-sulfonic acid (100%), (EDTA) (45%), and 1,10- phenanthroline (29%) but not by dinitro fluorophosphate (DFP) the inhibitor of alkaline proteases. Thus, it may be classified as a neutral protease.

Effect of Surfactants on Enzyme Activity

The effect of various surfactants on the activity of the purified protease at 50O C for 30 mins was shown in (Figure 4). At 1% of Triton-X-100, only less than 19.55% inhibition in activity was observed. While In the presence of an anionic surfactant, 0.1% sodium dodecyl sulfate (SDS), the enzyme retained nearly 83% activity and the residual activity of purified protease were 80.45%, 90.1%, 95% and 83%, respectively at 50^{O} C for 30 mins. It was observed that enzyme resisted against detergents at low concentrations of surfactants.

Tween 80 at 1% concentration enhanced the activity of the protease. In the presence of astrong ionic surfactant such as SDS (0.1%),the enzyme retained nearly 83% activity. Stability of alkaline protease from *B. stearothermophilus* AEAL2 towards the surfactant SDS gained importance in the light of reports that SDS had in general a strong inhibitory effect on proteases [37]. Combined effects of factors such as reduction in the hydrophobic interactions and the direct interactions with the protein molecule were believed to be the cause for the inhibition by SDS [38]. Cheng *et al*., [39] reported that the protease produced from *Bacillus alcalophilus* TCCC11004 was stable in 0.5 % SDS and retained 70.3 % of its initial activity after one hour of incubation.

A good detergent protease must be compatible and stable with all commonly used detergent compounds, such as surfactants, bleaches, oxidizing agents and other additives which might be present in the formulation [40].

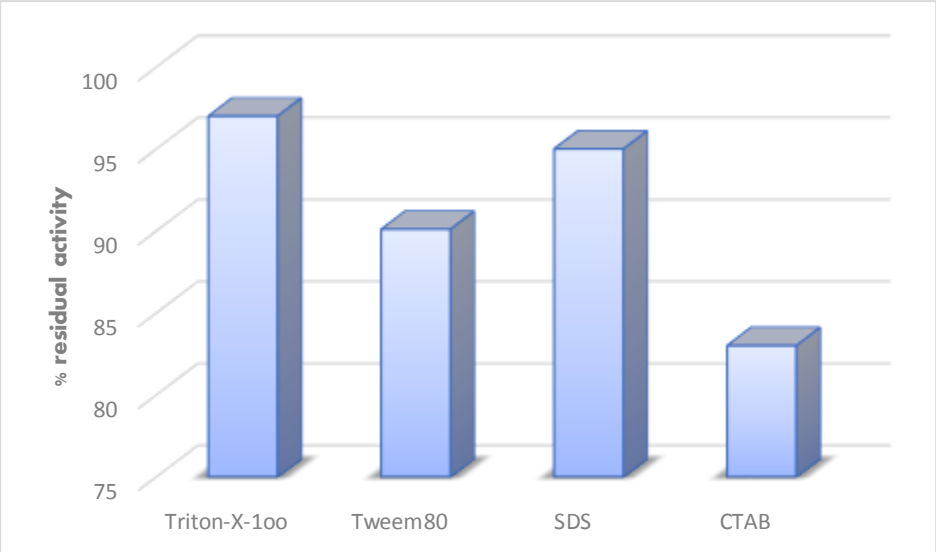

Figure (4): Surfactants effect on protease I activity produced by *B. stearothermophilus* AEAL2

The major application of alkaline protease is in detergent industry and it is always desirable for the enzyme to be stable in the presence of various detergent ingredients such as surfactants and bleaches. In addition to activity and stability at high temperature and pH ranges, a good detergent protease must be compatible and stable with all commonly used detergent compounds such as surfactants, bleaches, oxidizing agents and other additives which might be present in the formulation [41]. The purified alkaline protease from *B. stearothermophilus AEAL2* showed its activity and stability at high temperatures and pH range. The enzyme also showed its varied stability in the presence of different inhibitors, metal ions, a surfactant agent, indicating the possibility of commercial exploitation of the alkaline serine protease in the laundry detergent industry.

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