Research Article

Some Investigations on Protease Enzyme Production Kinetics Using *Bacillus licheniformis* BBRC 100053 and Effects of Inhibitors on Protease Activity

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Due to great commercial application of protease, it is necessary to study kinetic characterization of this enzyme in order to improve design of enzymatic reactors. In this study, mathematical modeling of protease enzyme production kinetics which is derived from *Bacillus licheniformis* BBRC 100053 was studied (at 37°C, pH 10 after 73 h in stationary phase, and 150 rpm). The aim of the present paper was to determine the best kinetic model and kinetic parameters for production of protease and calculating K_i (inhibition constant) of different inhibitors to find the most effective one. The kinetic parameters K_m (Michaelis-Menten constant) and V_m (maximum rate) were calculated 0.626 mM and 0.0523 mM/min. According to the experimental results, using DFP (diisopropyl fluorophosphate) and PMSF (phenylmethanesulfonyl fluoride) as inhibitors almost 50% of the enzyme activity could be inhibited when their concentrations were 0.525 and 0.541 mM, respectively. K_i for DFP and PMSF were 0.46 and 0.56 mM, respectively. Kinetic analysis showed that the Lineweaver-Burk model was the best fitting model for protease production kinetics DFP was more effective than PMSF and both of them should be covered in the group of noncompetitive inhibitors.

1. Introduction

Microbial proteases dominate the worldwide enzyme market, accounting for a two-thirds share of the detergent industry [1]. Microbial protease is one of the most important groups of industrial enzymes that caters to the requirement of nearly 60% of the worldwide enzyme market [2]. Of these, the alkaline proteases are of interest from a biotechnological perspective and find numerous applications in the food industry and meat tenderization process, peptide synthesis, infant formula preparations, baking, and brewing. Furthermore, they are used in pharmaceutical and medical diagnosis, in the detergent industry as additives, and in textile industry in the process of dehairing and leather processing [3, 4].

Currently, many economically important industrial enzymes are produced by cultivation of bacteria, such as *Bacillus* sp. [5, 6]. Over 300 tons of enzymes, mainly proteases, are being annually produced from *Bacillus* sp.

Proteases with high activity and stability in high alkaline range and high temperatures are interesting for bioengineering and biotechnological applications [7].

Kinetic study of enzyme constitutes the information pertaining to rates of activation and inactivation of enzymes and actually gives the rate at which a process occurs. The kinetic parameters controlling the rates of enzyme catalyzed reaction are incubation time, initial substrate concentration, initial enzyme concentration, pH, and reaction temperature [8].

Regarding previous studies, the optimized conditions for protease production, enzyme characterization, and enzyme immobilization were determined [9, 10]. Although there have been a number of studies on protease production by *Bacillus* species, little information on kinetic analysis of the protease production process is available in the literature. This research followed the study kinetic model for the production of protease enzyme based on experimental data. In this paper, the experiments were conducted to study the effects of various inhibitors on enzymatic activity of protease by determination of kinetic parameters and inhibition constants. Kinetic parameters of protease enzyme have been determined based on some *Bacillus* genus in which *Bacillus licheniformis* is less mentioned.

2. Materials and Methods

2.1. Bacterial Strain. The organism used was *Bacillus licheniformis* BBRC 100053 obtained from Biochemical and Bioenvironmental Research Center, a local culture collection in Sharif University Of Technology, Iran. The culture was maintained on nutrient agar medium at 30°C for 2 days and stored at 4°C [9]. Two inhibitors, DFP (diisopropyl fluorophosphate) and PMSF (phenylmethanesulfonyl fluoride), were obtained from Merck Co., Germany. All other reagents used were also of analytical reagent (AR) grade.

2.2. Inoculum Preparation and Protease Production. Inocula were prepared by adding a loop full of pure culture into 25 mL of sterile Luria-Bertani (LB) broth medium (composed of (g/L) peptone 10, yeast extract 5, and NaCl 5) [9]. Broth cultures were diluted with the appropriate broth to obtain 10^7 cfu/mL as estimated by absorbance at 600 nm. Target absorbance to obtain these populations was 0.2 for Gram-positive bacilli, based upon preliminary experiments [9–11].

About 8–10% inoculums from this culture were added to 100 mL Erlenmeyer flasks containing 20 mL production media containing lactose 1%, yeast extract 0.5%, peptone 0.5%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%, and pH 8 [9]. Media were autoclaved at 121°C for 20 min in 15 psi [3].

After incubation at 37°C for 73 h under shaking (150 rpm) the cultures were centrifuged and the supernatants were used for estimation of proteolytic activity. The growth of the microorganism was monitored by measuring absorbance at 660 nm. All experiments were carried out in duplicate.

2.3. Assay for Proteolytic Activity. Protease activity was determined by Anson's modified method using casein as substrate.

0.5 mL of enzyme solution was added to 4.5 mL of substrate solution (1% w/v, casein with 50 mM Tris-HCl buffer, and pH 8) and incubated at 30°C for 10 min. The reaction was stopped by adding 5 mL of 5% TCA (trichloroacetic acid) mixture (5% TCA, 9% Na-acetate, and 9% acetic acid) followed by 30 min holding at room temperature and centrifugation at 8000 rpm [12].

The precipitates were removed by filtration through Whatman-42 filter paper and absorbance of the filtrate was measured at 280 nm. One unit of protease activity was defined

as the amount of enzyme liberating 1 μg of tyrosine per min under assay conditions. Enzyme units were measured using tyrosine solutions (0–100 mg) as standard [13].

2.4. Determination of Kinetic Parameters (K_m , $V_{\max}$). Due to great commercial application of protease, it is necessary to study kinetic characterization of this enzyme in order to improve design of enzymatic reactors [14].

Enzymes are natural catalysts that speed up the chemical reactions. However, the speed of any fastidious reaction being catalysed by a particular enzyme can only reach a certain maximum value. This rate is known as $V_{\max}$ while K_m can be defined as the concentration of substrate at which half of the maximal velocity was obtained [15].

Michaelis-Menten equation:

$$V = \frac{V_{\max} [S]}{K_m + [S]} \quad (1)$$

Taking the reciprocal gives

$$\frac{1}{V} = \frac{K_m + [S]}{V_{\max} [S]} = \frac{K_m}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (2)$$

In order to investigate $V_{\max}$ and K_m , specific concentration of enzyme with different concentration of casein as substrate (0.25%, 0.45%, 0.65%, 0.8%, and 1%) (w/v), was prepared for identifying approximate values. The enzyme (0.5 mL) was added to 4.5 mL casein (1% w/v in 50 mM Tris-HCl buffer, pH 8) and the reaction mixture was incubated at 45°C for 10 min before the addition of 5 mL of TCA mixture (1.8% trichloroacetic acid, 1.8% sodium acetate, and 1.98% acetic acid). In different times (1, 2, 3, 4, and 5 minutes) the absorbance of the filtrate was measured at 280 nm. The slope of the plot of absorbance versus time was 0.1 to 0.4 using the best concentrations. The slope of the plot of absorbance (280 nm) versus time could determine ϵV_o ($\epsilon_{280} = 1.41/\text{cm} \cdot \text{mM}$).

2.5. Effect of Inhibitors on Protease Activity. Different inhibitor concentrations (volume ratio of inhibitor to enzyme) were used and increased up to inhibit more than 50% of the enzyme activity. For PMSF 0.025, 0.0075, 0.002, and 0.001 and for DFP 0.01, 0.005, 0.001, and 0.0004 volume ratios were selected and added at the beginning of exam. The reaction was started by adding 50 μL of enzyme solution to 450 μL of various substrates solutions. The reaction was stopped, by adding 50 μL of 5% TCA to this mixture, followed by filtration and measuring the absorbance at 280 nm. The slope of the plot of OD/ ϵ versus time determined V_o for different volume ratio (or inhibitor concentration) using various substrate concentrations (0.75%, 1%, 1.25%, and 1.5%).

The plot of $1/V$ versus $1/S$ identified the kind of group in which the inhibitors should be covered [16].

3. Results and Discussions

3.1. Kinetic Constant Calculation of Protease Enzyme Production. According to different ϵV_o for various initial substrate

TABLE 1: Substrate concentrations for calculating kinetic parameters.

Time (min)	OD (280 nm)		
	$S_1 = 1.5\%$	$S_2 = 2.25\%$	$S_3 = 3\%$
1	0.694	0.543	0.64
2	0.734	0.614	0.685
3	0.788	0.662	0.716
4	0.808	0.696	0.776
5	0.8	0.696	0.862

TABLE 2: The absorbance of the samples with different PMSF inhibitor concentrations.

X = (V_i/V_o)	Time (min)	OD (280 nm)			
		$S_1 = 0.75\%$	$S_2 = 1\%$	$S_3 = 1.25\%$	$S_4 = 1.5\%$
0.001	1	0.637	0.643	0.616	0.750
	2	0.652	0.658	0.675	0.812
	3	0.713	0.715	0.694	0.834
	4	0.739	0.760	0.767	0.924
	5	0.785	0.808	0.808	0.974
0.002	1	0.477	0.483	0.487	0.533
	2	0.514	0.525	0.533	0.540
	3	0.540	0.543	0.571	0.579
	4	0.578	0.600	0.619	0.675
	5	0.608	0.634	0.655	—
0.0075	1	0.371	0.374	0.375	0.379
	2	0.392	0.399	0.405	0.410
	3	0.407	0.416	0.424	0.430
	4	0.433	0.445	0.456	0.467
	5	—	—	0.477	—
0.025	1	0.256	0.258	0.259	0.259
	2	0.267	0.272	0.273	0.277
	3	0.272	0.276	0.280	0.284
	4	0.287	0.291	0.297	0.302
	5	—	—	—	—

concentrations, the Lineweaver-Burk model was selected as the best kinetic model and K_m and V_{max} were calculated by the plot of $1/V$ versus $1/S$. The obtained final ranges of initial substrate concentration were examined to identify amounts of ϵV_o (Table 1). Through the slope and the interception of the plot of $1/V$ versus $1/S$, the exact values of K_m and V_m were determined 0.626 mM and 0.0523 mM/min, respectively (Figure 1).

3.2. Inhibition Kinetics and Constant. The absorbance of the samples with different inhibitor concentrations and in the presence of several substrate concentrations was measured (280 nm) for DFP and PMSF, as shown in Tables 2 and 3.

Similar experiments were studied in the absence of inhibitors with respect to control (Table 4).

FIGURE 1: Kinetic constants of protease enzyme production (the Lineweaver-Burk plot).

TABLE 3: The absorbance of the samples with different DFP inhibitor concentrations.

X = (V_i/V_o)	Time (min)	OD (280 nm)			
		$S_1 = 0.75\%$	$S_2 = 1\%$	$S_3 = 1.25\%$	$S_4 = 1.5\%$
0.0004	1	1.065	1.074	1.079	1.084
	2	1.111	1.124	1.139	1.148
	3	1.151	1.179	1.191	1.204
	4	1.198	1.224	1.254	1.171
	5	—	1.281	1.308	—
0.001	1	0.669	0.676	0.680	0.685
	2	0.711	0.724	0.735	0.743
	3	0.745	0.864	0.781	0.792
	4	0.789	0.816	0.836	0.855
	5	—	—	—	—
0.005	1	0.374	0.378	0.381	0.377
	2	0.4	0.41	0.414	0.414
	3	0.419	0.419	0.439	0.439
	4	0.448	0.465	0.476	0.482
	5	—	0.491	—	0.515
0.01	1	0.234	0.237	0.238	0.239
	2	0.251	0.257	0.26	0.265
	3	0.26	0.267	0.274	0.28
	4	0.26	0.267	0.299	0.308
	5	—	—	—	—

Afterwards, different ϵV_o values were calculated from the slope of the plot of absorbance versus time. According to

TABLE 4: The absorbance of the samples in the absence of inhibitor concentrations.

Time (min)	OD (280 nm)			
	$S_1 = 0.75\%$	$S_2 = 1\%$	$S_3 = 1.25\%$	$S_4 = 1.5\%$
1	1.148	1.169	1.197	1.302
2	1.169	1.21	1.24	1.361
3	1.207	1.259	1.298	1.431
4	1.246	1.313	1.362	1.487
5	1.33	1.365	1.421	1.555

FIGURE 2: Determination of apparent kinetic parameters (K_m^{app} , V_m') for PMSF.

the plots of $1/V$ versus $1/S$ as shown in Figures 2 and 3 and various types of inhibitors, apparent kinetic parameters (K_m^{app} and V_m') were calculated (based on Lineweaver-Burk equation) for different inhibitor concentrations. Results showed that both inhibitors (PMSF and DFP) should be classified as noncompetitive inhibitors:

$$V = \frac{V_{\text{max}}^{\text{app}} [S]}{K_m + [S]} \quad V_{\text{max}}^{\text{app}} = \frac{V_{\text{max}}}{1 + ([I]/K_i)} \quad (3)$$

Secondary plot was used to determine inhibition constant (K_i). The slop of the plot and the intersection of the line with X axis showed an increase in $1/V_m'$ (V_{max}' and V_{max} were described as the maximum velocity in the presence and absence of inhibitors). As indicated in Figures 4 and 5, K_i obtained 0.56 and 0.46 mM for PMSF and DFP, respectively.

3.3. The Plot of IC_{50} . The specific concentration of inhibitors which was able to inhibit almost 50% of the enzyme activity was identified through Figures 6 and 7. In addition via the plot of enzyme activity ($V_i/V_o\%$) versus inhibitor concentration ($[I]$) V_i and V_o were identified as initial velocity

FIGURE 3: Determination of apparent kinetic parameters (K_m^{app} , V_m') for DFP. FIGURE 4: Determination of inhibition constant (K_i) for PMSF.

in the presence and absence of inhibitor. The inhibitor concentration met the required value when V_i/V_o was 0.5.

50% of the enzyme activity could be inhibited in the presence of 0.525 and 0.541 mM of DFP and PMSF, respectively (Figures 6 and 7). The identical enzyme activity should be intended for all steps.

4. Conclusions

In this paper, mathematical modeling of protease enzyme production kinetics derived from *B. licheniformis*, under optimum condition (37°C, pH 10), was studied.

FIGURE 5: Determination of inhibition constant (K_i) for DFP. FIGURE 6: IC₅₀ curve for PMSF (the effective concentration of PMSF for reducing enzyme activity up to 50%).

Based on the results, the Lineweaver-Burk model was the best fitting model for protease production kinetics. The kinetic parameters K_m , V_{max} were 0.626 mM and 0.0523 mM/min, respectively.

The results showed that DFP and PMSF were noncompetitive inhibitors. In order to reduce the enzyme activity up to 50% of its initial activity, the required concentrations of DFP and PMSF were 0.525 and 0.541 mM. In addition inhibition constant (K_i) is obtained 0.46 and 0.56 mM for DFP and PMSF, respectively.

FIGURE 7: IC₅₀ curve for DFP (the effective concentration of DFP for reducing enzyme activity up to 50%).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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