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Reactivity of Alkaline Protease to Keratin and Collagen Containing Substances

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ABSTRACT

The reactivity of partially purified alkaline protease from *Bacillus subtilis*, to keratin and collagen containing substances has been investigated. The experimentally obtained apparent values of the Michaelis-Menten constant (K_m), the maximum reaction rate (Kmax), and the energy of activation (Ea), lead to the conclusion that:

1. The keratin containing substrates are not so easily digested as those containing collagen.
2. The kinetics of the reaction with time has been investigated.

The relationship of Kmax was a function of temperature, aiming at the industrial application of this reaction, has been obtained.

Index Entries: Alkaline protease; waste matter; keratin; collagen; reactivity; kinetic.

INTRODUCTION

The protein deficiency on a national and global scale make the search for novel protein sources particularly important. Wastes rich in protein such as industrial poultry feathers, carrion, and hide scrap are suitable sources through technological processing employing proteolytic enzymes into protein concentrates for animal feed.

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The extracellular alkaline protease EC (3.4.2.1.4) of bacterial origin has been a widely investigated enzyme. The investigations has been usually carried out with a highly purified and inactivated enzyme, under gel chromatography and electrophoresis conditions (*1,7*). The enzyme purification methods are usually time and labor consuming. On the other hand, the industrial use of this enzyme requires data on the activity of the crude or the partially purified enzyme. The state of the enzyme is of importance from the stand point of its industrial utilization with processing keratin and collagen enriched waste matters in mind.

The aim of the present investigation was to examine the alkaline protease reactivity with respect to two kinds of such substances, obtained as waste water from the meat and tanning industries. Special attention has been paid to the reaction kinetics in order to specify the optimal conditions for its industrial application.

MATERIALS AND METHODS

Alkaline protease of bacterial origin was produced by the Factory for Enzyme Preparations in Botevtown, Bulgaria.

The crude preparation was dissolved in distilled water, and repeatedly purified through chromatography, over a cellulose 3.7×41 cm column until the eluant reached an absorption of 0.25 at 280 nm.

The enzyme partially purified in this manner, consisted of 80–85% proteins, 18–20% salts, dyes, carbohydrates and so on. Its activity after lyophilization was about 60 U/mg. protein, resulting from the presence of neutral protease (*8*). One unit corresponds to the amount of enzyme that libirates 1 μmol of Folin–positive–amino acids and peptides (calculated as tyrosine)/min at pH 7.5 and 27°C with hemoglobin as substrate.

The level of enzyme homogeneity as well as its mol wt were determined by SDS polyacrylamide gel-electrophoresis and subsequent measurement of the protein band mobility from the anode to the catode, according to the method of Möberg and Ferenström (*9*). The SDS electrophoresis was carried out in 10% acrylamide gels in 0.05 *M* phosphate buffer, pH 7.5 in a LKB-multiphore system, within approx six h. The protein component in the remainder of the gel was fixed by the method described (*10*). The following standards with indicated mol wt were used: ovalbumin /34,000/, carbon anhydrase /30,000/, bovine trypsin /24,000/, human trypsin /20,000/, fish myoglobin /17,000/, and horse cytochrome C /13,000/.

The protein substrate materials were industrial poultry feathers, designated respectively as PSAT-1 and PSAT-2 and carrion and scraps from the hide industry, designated respectively as KH–3 and KH–4.

The first two substrates are keratin rich, whereas the other two contain more collagen. The total protein content of the samples was between 60 and 75% of dry matter.

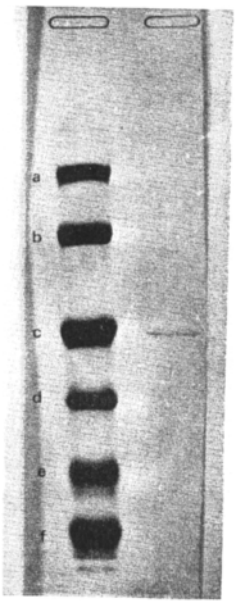

Fig. 1. SDS-gel experiments showing single protein band after electrophoresis of standards of known mol wt: a. ovalbumin (34,000), b. carbon anhydrase (30,000), c. bovine trypsin (24,000), d. human trypsin (20,000), e. fish myoglobin (17,000), and f. cytochrome (13,000).

The samples were solubilized in fine ground state through heating at 50–60° in 1*N* sodium hydroxide.

The absorption spectra of the enzyme and of the substrate were recorded in a buffer at pH 11 and in a 0.5 *M* solution of $CaCl_2$ and $MgCl_2$. A thermostated cuvet with a 1 cm light-path was used. The spectra were taken on a Specord 11-double beam spectrophotometer.

RESULTS AND DISCUSSION

The results obtained from isoelectrophocusing the enzyme preparation by SDS polyacrylamide gel-electrophoresis are shown in Fig. 1. The single polypeptide band, observed after partial purification, shows a good sample homogeneity. This confirms our assumption that the alkaline protease is stable under the conditions used for partial purification.

The average molecular mass was calculated to be $24{,}500 \pm 2{,}000$ on the basis of the data obtained from the electrophoretic mobility of the enzyme band and the respective standards.

This value is relatively low in comparison to the value obtained by other authors via the determination of the amino-acids content (*11*). One possible explanation of this fact could be found in the greater compactness probably, this results from the undisturbed native conformation of the used.

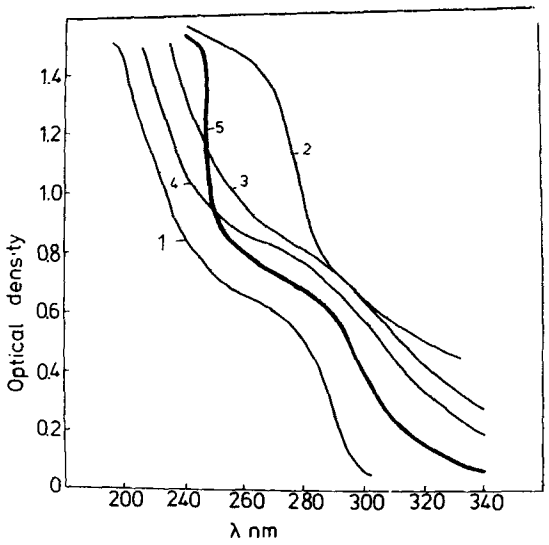

Fig. 2. Absorption spectra of alkaline protease and substances used as substrates: 1. PSAT-1, 0.3 mg/mL, 2. PSAT-2, 0.25 mg/mL, 3. KH-3, 0.4 mg/mL, 4. KH-4, 0.2 mg/mL, and 5. Alkaline protease, 0.005 mg/mL. All spectra were recorded in buffer pH=11 at 25°C.

We investigated the kinetic properties of the alkaline protease with respect to four substrates, i.e., PSAT-1, PSAT-2, KH-3 and KH-4. Fig. 2 gives the absorption spectra of the pure enzyme and the substrates. The data presented show that the absorption of the enzyme and the substrates are very close and can be characterized by a wide shoulder in the absorption band within 240 and 300 nm. We therefore worked with the differential absorption spectrum representing the difference between the enzyme's absorption spectrum and that of the substrate. This manner determined the wavelength maximum of the enzyme obtained in the presence of a given substrate.

It is known that kinetically, partially purified alkaline protease exhibits a high proteolytic activity to various protein-containing substrates. Most probably, this results from the undistured native conformation of the active site in the partially purified alkaline protease as well as to the presence of neutral protease (*8*).

Enzyme kinetics theory describes such reactions by the following equation:

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} ES \xrightarrow{K_{+2}} E' + P \qquad (1)$$

At S>>E, the relative change of S with time is comparatively slow. Thus, the overall reaction rate for the steady-state system described with Eq. 1 will be:

$$Vov = - K_{+2}[ES]$$

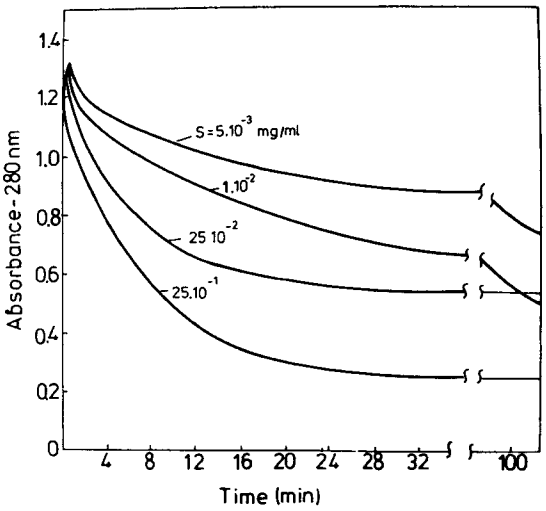

Fig. 3. Time course of absorbancy change at 280 nm. during the reaction of Alkaline protease with PSAT-1 at different initial concentrations. Conditions were the same as those in Fig. 2.

It is impossible to determine [ES] using the classic investigation methods. By spectrophotometry, it is possible however to estimate the accumulation of the recovered enzyme E′ by measurement of the absorption at 280 nm. The Michaelis-Menten constant, showing the enzyme-substrate affinity, can be experimentally determined from the equation for the double reciprocal values that, in this case, can be given as:

$$1/Kobs = Km/Kmax.\ 1/S + 1/Kmax \quad (2)$$

Kobs is the apparent pseudo-first order rate constant that reflects the rate of accumulation of E′ with time. Practically, Kobs depends on the substrate concentration and can be experimentally determined from the kinetic curves in Fig. 3. For this purpose, the experimentally determined points in the change of absorption with time at 280 nm can be replaced in the equation:

$$At = A_\infty\ (1 - e^{-Kobs.t}) \quad (3)$$

where At is the absorption with time, A_∞ is the absorption value at the end of the reaction. The experimentally estimated values of Kobs were determined with a ±10% accuracy.

The second equation for the substrate PSAT-1 is presented in Fig. 4, for temperatures in the interval of 25–85°C. The double reciprocal plots and Kobs against the substrate concentrations were straight lines in all cases. The respective K_m can be calculated from the slopes of the lines, knowing Kmax, i.e., Kobs extrapolated to infinite substrate concentration. The results derived from this plots for t° = 25°C are listed at Table 1. The dimensions of the Michaelis-Menten constant are not comparable to these of the classical one, but it gives a clear idea about the enzyme-substrate affinity in this particular case. The apparent K_m values are of same

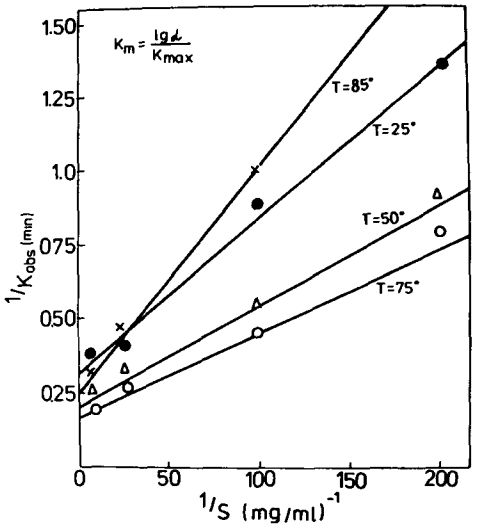

Fig. 4. Double reciprocal plots of Kobs against concentration of PSAT-1 at different temperatures. The initial enzyme concentration was 0.005 mg/mL in buffer, pH 11.

Table 1
The Apparent Constants K_m and Energies of Activation Ea for Reaction of Alkaline Protease with Keratin and Collagen Containing Preparations[a]

Preparation used as substrate	Concentration range (mg/mL)	$K_m \cdot 10^{-3}$ (mg/mL)	$1/K_m$	Ea (Kcal)
1. PSAT-1	5.10^{-3}-25.10^{-1}	15	66	3.2±0.6
2. PSAT-2	1.10^{-3}- 2.10^{-1}	27	37	2.8±0.1
3. KH-3	1.10^{-3}- 1.10^{-1}	45	22	1.7±0.10
4. KH-4	8.10^{-3}- 3.10^{-1}	57	14	1.5±0.15

[a]The values were determined at 5.10^{-5} mg/mL enzyme in buffer, pH 11 at 27°C.

order and are arranged in an ascending order meaning that the enzyme-substrate affinity (1/ K_m) decreases in the same way. Notice should be taken, that the way K_m has been determined applies for conditions of a stationary state system, whereas the experimental values of Kobs apply mainly for the initial reaction stages. As Fig. 3 shows, the absorption change with time apparently slows down in 40 min and tends to constancy. This is an indication that, from this moment onward, the enzyme reaction becomes time-dependent.

If such reactions are to be applied on an industrial scale, one should necessarily know not only the enzyme-substrate affinity (1/ K_m) specific parameters, but also those determining the energetics of the process as well. Therefore, the Kmax values were determined for the respective tem-

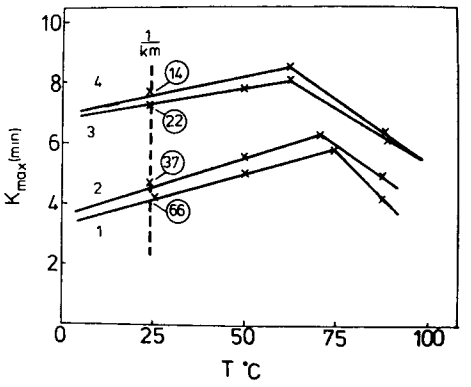

Fig. 5. Temperature dependence of the maximum reaction rate Kmax for reaction of alkaline protease with substances used as substrates. The initial concentrations of reagents were the same as those in Fig. 2: 1. PSAT-1, 2. PSAT-2, 3. KH-3, 4. KH-4.

peratures. Figure 5 illustrates this relationship. Again, one can see the typical enzyme reaction mode. The temperature optimum for PSAT-1 and PSAT-2 substrates is at 75°C, whereas for the samples KH-3 and KH-4, it is at 60°C. In agreement with the Michaelis-Menten theory, when the substrate affinity, i.e., $1/K_m$ is higher, Kmax has lower values and vice versa. The rise in temperature favors the formation of recovered enzyme until the respective temperature maximum is reached. The temperature maxima's difference between the two substrate types is 15°C. This implies that there is a difference in the apparent activation energies in both cases. Such an assumption can be easily shown to exist by the equation, that, in our case, assumes the form of:

$$\log \text{Kobs} = \text{const} - \text{Ea/RT} \quad (4)$$

Now it is possible to calculate the respective Ea for the different substrates (Table 1). It can be seen that almost a 50% difference exists between the activation energies in the two cases.

The present results may be summarized as follows: The partially purified alkaline protease from *Bacillus subtilis* can be applied for the digestion of keratin and collagen containing substrates. Under the conditions of the reaction described above, the substrate degradation takes place at a measurable rate. The process runs in two stages: a fast initial stage, that is completed in 40 min, and a second—a slow one, that takes more than one hour.

The extent of the reaction of the alkaline protease with the studied substrates can be evaluated by determining via spectrophotometric measurements the rate with which the recovered enzyme is formed. The obtained spectral data, treated in accordance with the Michaelis-Menten theory, allow an empirical estimation of the reactivity of the alkaline protease with respect to the studied substrates to be drawn.

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