Comparison of Alkaline Proteinase from Hyperproductive Mutants and Parent Strain of *Aspergillus sojae*

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Physico-chemical properties of alkaline proteinase from the parent strain were compared with those from hyperproductive mutants of *Aspergillus sojae*. All the results on behavior of enzyme protein to ion exchange resin and celluloses, gel filtration, ultracentrifugal sedimentation, disc electrophoresis and isoelectrofocusing on polyacrylamide gel column, specific activity, substrate specificity, and kinetic constants provided evidence in favor of the conclusion that the parent and mutant strains produced the chemically identical enzymes and that superactivity of alkaline proteinase in culture extracts or filtrates of mutant strains was not attributed to alteration of catalytic property of the enzyme, but to hyperproduction of the identical enzyme resulting from the genetic change in the regulatory mechanism of enzyme synthesis.

In a previous paper¹⁾ several mutants exhibiting hyperproduction of proteinase were successively derived from strain X816 of *Aspergillus sojae* by ultraviolet irradiation and isolated by the use of the rapid and convenient screening method.²⁾ The proteinase activity of these mutants reached up to 6 times much as the level of ordinary strains.

Strain X816 was also induced from strain K.S. of *Aspergillus sojae* by X-ray irradiation and was able to produce proteinase twice as much of the original strain.³⁾ As to the nature of hypersynthesis of the enzyme by strain X816, Oda⁴⁾ presented the biochemical evidence that the enzyme from the mutant strain appeared to be identical to that from original strain.

At least, two mechanisms are considered to

explain hyperactivity of proteinase in mutant strains. Yanofsky *et al.*⁵⁾ reported that the changes of tryptophan synthetase activity in mutant strains of *Escherichia coli* was attributed to the alteration of chemical structure of the enzyme protein caused by mutation in a structure gene. On the other hand, Jacob and Monod⁶⁾ isolated a number of mutants of *E. coli* possessing different activity of β -galactosidase and deduced by genetic studies that the activity of this enzyme was dependent on the amount of the enzyme which was regulated by a promotor site. The mechanism of hyperactivity of the enzyme from mutant strains of *Aspergillus sojae* is of particular interest in relation to gene function whether the expression of hyperactivity in the mutant strains is the result of mutation in a structure gene or in a promotor site if the principle of control mechanism reported in bacteria is also applicable to fungi.

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MATERIALS AND METHODS

Cultural conditions. Induced mutants, *Aspergillus sojae* X816, U34, and U108, were isolated and maintained in this laboratory.^{1,3)} Wheat bran koji cultures were initiated in 500 ml Fernbach flasks by inoculating one hook of conidia from a 7 day-old slant into 50 g wheat bran moistened with 35 ml water and incubated at 30°C for 70 hr.

Purification of enzyme. The purification of alkaline proteinase from strains U34 and U108 was performed by the method of Hayashi *et al.*⁷⁾ with following modifications (Fig. 1). In batchwise treatment with ion exchange resin, Duolite CS101 was replaced by Amberlite IRC50 equilibrated with 0.05 M acetate buffer at pH 4.8.

The enzyme fractions passed through DEAE cellulose column was dialyzed against 0.01 M acetate buffer at pH 5.0 and further applied to a column (3 × 30 cm) of CM cellulose equilibrated with 0.01 M acetate buffer (pH 5.0). Elution was obtained by a linear gradient of sodium chloride concentration in 0.01 M phosphate buffer at pH 7.0. The flow rate was adjusted to 90 to 100 ml/hr and 10 ml fractions were collected. Pooled

fractions showing enzyme activity were desalted by dialysis and lyophilized for gel filtration. Partially purified alkaline proteinase from strain X816 was kindly supplied by Mr. K. Hayashi.

Enzyme assays. Proteolytic activity measurements were carried out by a modification of the Anson's method.⁸⁾ Each 1 ml of enzyme solution and water was added to 1 ml of 1.5% milk casein (Hammersten) in 0.15 M phosphate buffer at pH 7.3. After incubation at 30°C for 10 min, the reaction was terminated by the addition of 5 ml of 7.5% trichloroacetic acid and the precipitate formed was filtered off. To 1 ml of the filtrate was added 4 ml of 0.5 M sodium carbonate, followed by instant mixing with 1 ml of phenol reagent⁹⁾ and the optical density of developed color was determined at 660 m μ . One unit of alkaline proteinase (PU) was defined as the amount of the enzyme which liberates 1 μ g of tyrosine in the reaction mixture at 30°C and pH 7.3 per one min under the above condition. In experiments to determine specific activity of parent and mutant enzymes, 1.5% soybean α -protein (Nutritional Biochemicals Corporation U.S.A.) was also used as the substrate for proteolysis.

The esterase activity was measured spectrophotometrically according to the method of Schwert and Takenaka.¹⁰⁾ The reaction mixture contained 1 mM N-benzoyl-L-arginine ethyl ester (BAEE) in 0.1 M borate buffer (pH 8.0) containing 5 mM calcium chloride and 0.2 mg enzyme in a total volume of 3.2 ml. BAEE was obtained from Mann Research Laboratories, U.S.A.

Ultracentrifugation. Sedimentation analysis was performed on a Hitachi model UCA-1 analytical centrifuge at 55,430 rpm at 20°C in a single sector cell.

Electrophoretic studies. Disc gel electrophoresis was carried out with polyacrylamide gel according to the method of Davis¹¹⁾ for pH 9.4 gel and of Reisfield *et al.*¹²⁾ for pH 4.0 gel.

FIG. 1. Purification Scheme of Alkaline Proteinase from *Aspergillus sojae*.

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In a standard run 100 μ g of the protein sample was added to the column as a suspension in an equal volume of 40% sucrose and electrophoresis was performed at room temperature for 60 min at 5 mA per gel column. Protein bands were localized by staining the gels in 1% solution of amidoschwartz prepared in 7% acetic acid. Destaining was done by washing repeatedly in 7% acetic acid.

A micro-method of isoelectrofocussing in polyacrylamide gel was performed according to the method of Catsimpoolas.¹³⁾ The protein sample, calculated to give 0.5 mg protein per gel column, was dissolved directly in the reagents to prepare gel. After 85 min of the isoelectrofocussing at 95 V, the pH range was determined by slicing an unstained gel column and suspending the sliced section in 1.0 ml of distilled water for 4 hr. The pH is measured at 20°C with a microelectrode. Alkaline proteinase in the extracts of the above sliced gel was assayed after appropriate dilution with 0.15 M phosphate buffer at pH 7.3. The activity of the enzyme was expressed as optical density at 275 m μ of the filtrates soluble to trichloroacetic acid, since unknown ingredient(s) of Ampholine interfere the use of the phenol reagent in the Anson's method.

RESULTS

Enzyme purification

Since most of the alkaline proteinase applied to a column (3 $\times$ 30 cm) of DEAE cellulose equilibrated with 0.01 M phosphate buffer at pH 7.0 was found to pass through the column, the passed fractions were again adsorbed on a column of CM cellulose after dialysis. The enzyme from both strains U34 and U108 were eluted at the concentration of 0.5 M of sodium chloride. The peaks of enzyme activity were almost superimposed with those of proteins. The fractions of these peaks were collected, desalted by dialysis, and then lyophilized.

The lyophilized preparation obtained above was dissolved in a small volume of 0.02 M ammonium acetate buffer (pH 6.0) and applied to a column (2 $\times$ 90 cm) of Sephadex G-100 equilibrated with the same buffer. As shown in typical elution profiles (Fig. 2), all the

FIG. 2. Gel Filtration of Alkaline Proteinase from Strain X816, U34, and U108 of *Aspergillus sojae* through Sephadex G-100.

○—○ O.D.₂₈₀, ●—● proteinase

enzymes from strains X816, U34, and U108 appeared in almost the same fractions and elution patterns for these strains were very similar. The proteolytic fractions were collected, desalted and lyophilized. These preparations were used for further experiments to identify whether or not mutant enzymes were identical to the parent enzyme.

Sedimentation analysis

The sedimentation coefficients of 0.37% solution of alkaline proteinase from parent and mutant strains in 0.1 M sodium chloride and 0.01 M phosphate buffer were measured at 55,430 rpm in a Hitachi Model UCA-1 ultracentrifuge. They were 3.0, 2.7, and 3.4 for the enzymes from strains X816, U34, and U108,

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FIG. 3. Ultracentrifugal Analysis of Purified Alkaline Proteinase from *Aspergillus sojae*.

Runs were performed at a rotor speed of 55,430 rpm. Samples were dissolved in 0.01 M phosphate buffer at pH 7.0 containing 0.1 M sodium chloride. Photographs were taken at every 23 min.

respectively. The similarity of sedimentation coefficients and patterns (Fig. 3) for three enzymes suggests that the enzymes from mutant strains may be identical to that from the parent strain.

Disc gel electrophoresis

The electrophoretic movement in polyacrylamide gel of the enzymes from three strains were completely coincided each other at the pH of acid or alkaline (Fig. 4). The results of electrophoresis besides to the sedimentation analysis gave the evidence of physical homogeneity of the enzyme preparations.

The mutant enzymes also gave very similar profiles of isoelectrofocusing to that of parent enzyme (Fig. 5). Isoelectric points of the enzyme proteins obtained by this method were 5.26, 5.19, and 5.25 for strains X816, U34, and U108, respectively. These values were very close to the isoelectric point of alkaline proteinase from strain K.S. of *Aspergillus sojae* determined by the other method.¹⁴⁾

The above electrophoretic properties were further evidence to support the presumption

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FIG. 4. Polyacrylamide Gel Electrophoresis of Purified Alkaline Proteinase from *Aspergillus sojae* at pH 9.4 and 4.0.

1 and 1': strain X816, 2 and 2': strain U34, 3 and 3': strain U108.

that two mutant enzymes might be chemically identical to the parent enzyme.

Specific activity and substrate specificity

Specific activity of the enzyme from the parent strain for milk casein, soybean α -protein, and BAEE were compared with those from mutant strains in order to find whether or not there was some difference in substrate specificity among the parent and mutant enzymes (Table I). These results again indicate that there might not be any difference in chemical structure of the enzymes from test strains.

Kinetic constants

To confirm the hypothesis that the active

FIG. 5. Isoelectrofocusing Patterns of Purified Alkaline Proteinase from Strains X816, U34, and U108 of *Aspergillus sojae*.

○—○ proteinase, ●—● pH

TABLE I. SPECIFIC ACTIVITY AND SUBSTRATE SPECIFICITY OF ALKALINE PROTEINASE FROM THE PARENT AND MUTANT STRAINS OF *Aspergillus sojae*

Strain	Milk casein		Soybean α -protein		BAEE	
	units ^{a)}	%	units ^{a)}	%	units ^{b)}	%
X816	688	100	187	100	0.50	100
U34	788	117	193	103	0.54	109
U108	819	122	209	112	0.50	100

^{a)} μ g tyrosine/min/mg protein

^{b)} μ mole N-benzoyl-L-arginine/min/mg protein

centers of alkaline proteinases from mutant strains might be identical to that from the parent strain, the apparent Michaelis constants

TABLE II. KINETIC CONSTANTS OF ALKALINE PROTEINASE FROM THE PARENT AND MUTANT STRAINS OF *Aspergillus sojae* ACTED ON MILK CASEIN

Strain	K_m (%)			V_{max} (min ⁻¹)		
	pH 7.3 ^{a)}	pH 8.0 ^{b)}	pH 10.5 ^{c)}	pH 7.3 ^{a)}	pH 8.0 ^{b)}	pH 10.5 ^{c)}
X816	0.286	0.393	1.00	46.5	57.2	125.0
U34	0.278	0.410	0.91	47.6	62.5	105.2
U108	0.301	0.400	1.11	47.0	60.6	111.0

^{a)} 0.15 M phosphate buffer

^{b)} 0.1 M veronal buffer

^{c)} 0.067 M carbonate buffer

(K_m)¹⁵⁾ and the maximum velocities (V_{max}) of the enzymes were determined at pH 7.3, 8.0, and 10.5 by the Lineweaver-Burk plots.¹⁶⁾

Since the variation of the both K_m and V_{max} under the same conditions seemed to be within experimental errors (Table II), it could be concluded that all the enzymes had the same affinity to the substrates. In other words, chemical structure of the active centers of mutant enzymes might be unaltered by mutation.

DISCUSSION

The above results on gel electrophoresis and isoelectrofocusing on polyacrylamide gel columns and sedimentation analyses indicate the physical homogeneity of the enzyme preparations. In addition to these results, very similar behavior of alkaline proteinase in chromatography of ion exchange resin and celluloses and in gel filtration and the data of the substrate specificity, specific activity, and kinetic constants provide evidence in favor of the conclusion that the enzymes from three different strains are physico-chemically identical each other.

Thus, the hyperactivity of alkaline proteinase in the culture extracts or filtrates of mutant strains must ascribe to hyperproduction, but

not to the alteration of chemical structure of the parent enzyme.

As to the mechanism of enzyme hypersynthesis, Horiuchi *et al.*¹⁷⁾ presented genetic evidence that it was attributed to the extra chromosomal segments carrying the special region of the concerned enzyme in case of the hypersynthesizing strain of *E. coli*, which can produce β -galactosidase at rates up to four times that of wild type strains.

Another mechanism to control enzyme synthesis in microorganisms is an operator-promotor theory proposed by Jacob and Monod.⁶⁾ In this model a promotor region is essential for expression of the operon and a genetic change in this region severely affect enzyme synthesis.¹⁸⁾ Arditti *et al.*¹⁹⁾ isolated a series of promotor mutants which had single base substitution and showed that point mutation in the promotor region dramatically changed the level of the enzyme synthesis.

Yamaguchi *et al.*²⁰⁾ also reported biochemical and genetic evidence to support the presence of such a regulator gene controlling the rate of α -amylase synthesis in *Bacillus subtilis*, which is linked to the structure gene.

It is still premature to conclude which

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mechanism mentioned above is effective to explain the ability of mutant strains of *Aspergillus sojae* producing high levels of alkaline proteinase. Further studies will be done to find the presence in the fungus of the extra chromosomal segments or of the regulator gene such as a promotor site in the lactose operon of *E. coli*.

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