DIFFERENTIATION OF ALKALINE PROTEASES FROM BACILLUS SPECIES

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A series of highly purified Bacillus alkaline proteases have been examined to determine whether a whole range of species and strains produce different alkaline proteases. The results indicate that there are only two types of alkaline proteases produced and further differentiation could not be made with the methods used.

Differences between microbial alkaline and neutral proteases have been described (1), but the differences between alkaline proteases produced by closely related organisms such as different species or strains of Bacillus are less well documented (2-5).

A number of Bacillus alkaline proteases (Subtilisins or Subtilopeptidases) have been isolated from fermentation beers, or crude enzyme products, or obtained commercially. The techniques for comparing enzymes have been applied to the differentiation of these enzymes and indicate that only two types of alkaline protease are produced.

EXPERIMENTAL

Proteases were isolated from crude enzyme isolates by chromatography on Duolite C-10 ion-exchange resin by a modification of the method of Hagihara (6), followed by reprecipitation, dialysis and lyophilization. When amylase and excessive pigment were present in the starting material, prior to chromatography amylase was removed by careful acetone fractionation in the presence of 2% calcium acetate w/v and then most of the pigment and the residual amylase removed by batch treatment with DEAE-cellulose (acetate form, pH 7.0). Crystalline proteases from B. amyloliquefaciens

(Nagarse), B. subtilis var amylosacchariticus, and B. subtilis (Subtilisins Novo and Carlsberg) were obtained from the Nagase Company, Osaka, Japan; Miles Laboratories, Inc.; and Novo Industri S/A, Copenhagen, Denmark respectively. The crude enzyme mixture produced by B. subtilis NRRL B3411 was obtained from Inorganic Research Department, Monsanto Company, St. Louis, Missouri. Crude enzyme mixtures from organisms tentatively identified as B. licheniformis and B. pumilis were isolated from laboratory shake-flask cultures by acetone precipitation. The purity (molarity of active enzyme) was determined by titration with cinnamoylimidazole (7). Protease activity was determined by the method of Anson (8) using casein as substrate. Esterolytic activity was determined using a series of CBZ-amino acid p-nitrophenyl esters (Cyclo Chemical Corp., Los Angeles, California) under pseudo-first-order conditions by measuring the absorbance change at 340 mμ with a Cary 14 recording spectrophotometer. The first order rates were determined from the curves graphically by the method of Kezdy (9). Antiserum was prepared by injecting the alkaline protease (8 mg. mixed with Freunds adjuvant) into mature rabbits. Bleeding was commenced after the fourth weekly injection.

RESULTS AND DISCUSSION

A flow sheet for the purification of one of the enzymes (from B. subtilis NRRL B3411) is shown in Table I. The enzyme was homogeneous as demonstrated by disc electrophoresis and gel-filtration chromatography. The other alkaline proteases were isolated similarly or were commercially available crystalline enzymes. The active enzyme component was determined by direct titration with cinnamoylimidazole to be 28-50% in all cases, assuming a molecular weight of 27,400. The enzymes were inhibited by DFP, but unaffected by 10^{-3} M EDTA. Autolysis makes further purification difficult

Table I

The Purification of B. subtilis NRRL B3411 Alkaline Protease

Fraction	Weight or Volume	Total Amylase Units	Total Protease Units, pH 7.0	Total Protease Units, pH 10.0
Crude mixture	100 g	32×10^6	136×10^6	42.0×10^6
50-67% Acetone ppt	470 ml	0	60.0×10^6	21.6×10^6
DEAE-cellulose filt.	450 ml	0	50.0×10^6	20.6×10^6
Duolite C-10 Eluate	1500 ml	0	14.4×10^6	13.8×10^6
Lyophilized powder	1.92 g	770	7.7×10^6	8.4×10^6

and also makes the fingerprint technique for examination of tryptic digests of the enzymes rather suspect as a method of differentiating proteases (10). The molecular weights and broad peptidase specificity do not provide any differentiation. The methods which have been found most useful in differentiating the alkaline proteases are (a) the ratio of esterase to protease activity, (b) the amino acid composition, and (c) serological cross-reactions. These methods were applied to a series of Bacillus alkaline proteases and the results summarised in Table II show that the enzymes form two distinct groups. The Subtilisin Carlsberg, B. licheniformis and B. pumilis enzymes comprise one group (Subtilopeptidase A), while Subtilisin Novo, Nagarse, B. subtilis NRRL B3411 and B. subtilis var amylosaccharitícus enzymes form a second group (Subtilopeptidase B). The "A" group of enzymes is characterised by a higher esterase to protease activity ratio, a lower serine and higher threonine and arginine content than the "B" group of enzymes. The two groups do not cross-react serologically, but enzymes within each group cross-react with antisera to the other enzymes of that group, and Ouchterlony plate patterns and quantitative precipitation reactions indicate quantitative identity of the members of the group.

Table II

The Classification of Bacillus Alkaline Proteases

Bacillus Species	subtilis	lichen-iformis	pumilis	subtilis var amylo-sacchariticus	subtilis	amylo-liquefaciens	subtilis NRRL B3411
Enzyme	Subtilisin Carlsberg	-	-	-	Subtilisin Novo	Nagarse	-
Protease, 10^6 u/g pH 10.0	5.30	5.60	6.05	3.90	4.70	4.20	5.15
Puirty, %	43.0	44.6	44.6	28.8	44.5	43.0	44.1
ARG[1]	4	4	5	3	2	2	2
THR[1]	19	20	21	15	13	13	13
SER[1]	32	32	31	39	37	37	37
CBZ-gly PNE[2]	29.9	25.1	26.5	13.7	13.3	11.8	12.9
CBZ-leuc PNE[2]	46.3	42.0	51.6	16.8	14.5	12.2	11.5
Anti-B. subtilis NRRL B3411[3]	-	-	-	+	+	+	+
Anti-subtilisin Carlsberg[3]	+	+	+	-	-	-	-

1. Moles amino acid assuming molecular weight to be 27,400.
2. Esterase activity expressed as 10^2 k/E, sec^{-1} $(mg/ml)^{-1}$.
3. Cross reaction with rabbit antisera on Ouchterlony plates.

Apparently the names Subtilisin and Subtilopeptidase have more historical than taxonomic justification and the nomenclature Bacillopeptidase (cf. Aspergillopeptidase) would probably be a more accurate classification.

Studies on fermentation beer or on crude precipitated enzymes from these organisms and a series of less well characterized Bacilli shows that organisms which produce alkaline proteases of type A apparently produce little if any neutral protease or amylase, while those producing type B alkaline proteases also produce comparable levels (on a unitage basis) of neutral protease and amylase.

In view of the increasing interest in Bacillus proteases, these observations on the various Bacilli at the molecular level should assist in the identification of enzymes and in the study of taxonomic classification of the Bacilli and the effects of mutations on protease production.

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