NEUTRAL PROTEASES OF THE GENUS BACILLUS

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Neutral protease of B. subtilis NRRL B3411 has been highly purified by chromatography on hydroxylapatite, and compared with B. subtilis var. amylosachariticus neutral protease and thermolysin. On the basis of activity against macromolecular substrates no differentiation was possible. Immunochemical and stability studies as well as amino acid composition distinguished thermolysin from the other proteases which were indistinguishable.

Although microbial neutral proteases have been well differentiated from the alkaline proteases (1), and some specificity comparisons made (2), they have not been grouped or differentiated as have the alkaline proteases (3). It has been claimed that the neutral protease of B. subtilis var. amylosachariticus (4) is different from the B. subtilis neutral protease (5), altho both enzymes apparently have the same specificity (4, 6, 7) as thermolysin (B. thermoproteolyticus enzyme) (1, 2, 8). The analogous situation of the amylases, especially the thermostable amylase produced by a thermophilic Bacillus (9), has already been questioned (10) and it seems that a detailed examination of the Bacillus neutral proteases would be timely.

EXPERIMENTAL

Isolation of the B. subtilis NRRL B3411 Neutral Protease

The starting material was a crude enzyme mixture obtained from Inorganic Chemicals Division, Monsanto Co., St. Louis, Missouri. The mixture also contained an α-amylase, alkaline protease and pigment. Since the α-amylase also adsorbed onto hydroxylapatite and eluted with the neutral protease, the amylase was removed prior to chromatography by either of the following methods:

a) Pigment was removed from a solution of the crude enzyme by batch treatment

with DEAE-cellulose (acetate form, pH 6.5). The protease was concentrated by ammonium sulfate fractionation (0.45 - 0.65 saturation). Residual amylase was removed by stirring with pulverised wheat starch in the presence of 12% ethanol. After reprecipitation of the enzyme in the filtrate by acetone, the enzyme was dissolved in 0.1% calcium acetate and dialysed at 5° against 0.1% calcium acetate b) Protease was isolated essentially free of amylase by solvent fractionation in the presence of 2% calcium acetate w/v. The 50-67% acetone precipitated fraction was redissolved in 0.1% calcium acetate solution and treated batchwise with DEAE-cellulose (acetate form, pH 6.5) to remove pigment.

The amylase-free solution of proteases in 0.1% calcium acetate solution was applied to a column of hydroxylapatite (BioRad) in 0.1% calcium acetate at 5° and washed with 0.1% calcium acetate solution. After the unadsorbed alkaline protease and some impurities had passed through the column, some inactive protein was eluted with 0.01M potassium phosphate pH 7.2, and then the neutral protease was eluted with 0.045M (or stronger) potassium phosphate pH 7.2. Phosphate was removed from the eluted protease by addition of solid calcium acetate 4% w/v while the pH was maintained at 7.0 by addition of N/1 NaOH solution. After dialysis against 0.1% calcium acetate solution the enzyme could be further purified by retreatment with DEAE-cellulose to remove last traces of pigment, or passage through a column of G-100 Sephadex prior to lyophilisation.

Other Materials and Methods

B. subtilis var. amylosaccharitícus neutral protease and *B. thermoproteolyticus* protease (thermolysin) were obtained from Miles Laboratories, Elkhart, Indiana and Daiwa Kasei Company, Osaka, Japan respectively and were used without further purification (other than dialysis in certain experiments). Protease activity was determined by a modification of the method of Anson (11) using casein as substrate. FAGLA (furylacryloylglycylleucine amide) activity was determined by the method of Feder (12).

Antiserum to enzymes was prepared by injecting the enzymes (8 mg, mixed with Freunds adjuvant) into mature rabbits. Bleeding was commenced after the

third weekly injection. Amino acid composition was determined on dialysed enzyme preparations with extrapolation to zero hydrolysis time for the serine and threonine values. Molecular weights were determined in the ultracentrifuge after dialysis against the reference buffer. Zinc was determined by atomic absorption spectroscopy.

RESULTS AND DISCUSSION

The hydroxylapatite elution pattern of the B. subtilis NRRL B3411 neutral protease is shown in fig. 1, and a flow sheet for a purification is shown in table I. This method of isolation has been found to be more reproducible than methods using ion-exchange resins.

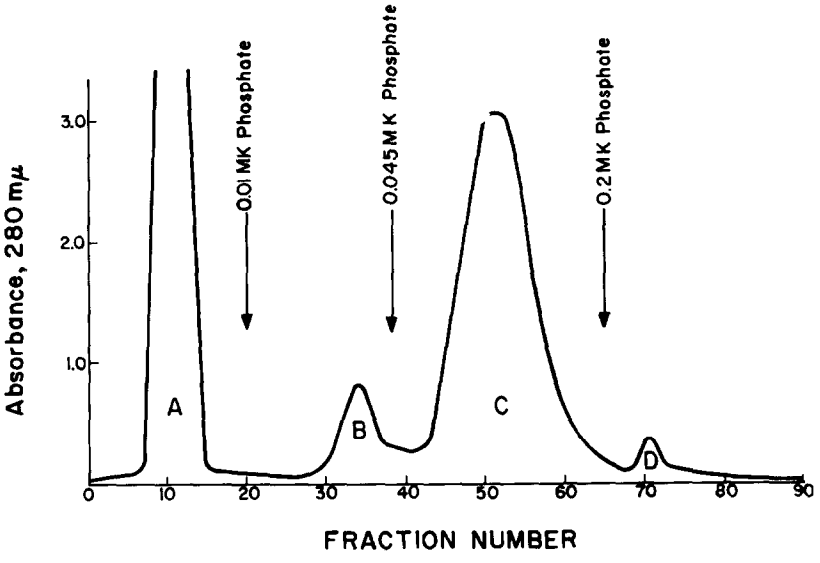

FIG. 1 Hydroxylapatite Chromatography of NRRL B3411 Neutral Protease. Amylase-free material from 5g crude enzyme mixture was applied to a 1.8 x 6.0 cm column at 5°, fractions of 5 ml were collected.

Comparative assay and analytical data on the NRRL B3411, amylosachariticus enzymes and thermolysin is shown in table II and clearly no distinction can be made on the basis of molecular weight, or activity against macromolecular substrates. Thermolysin has a higher activity with FAGLA substrate than the other two enzymes, and also a much higher heat stability in addition to possess-

TABLE I

Purification of the NRRL B3411 Neutral Protease

Fraction	Volume (ml)	Protein mg/ml	Total Amylase units	%	Total Protease units	%	Specific Activity, protease units per mg protein
Starting extract	200	59	600,000	100	2.54$\bar{M}$	100	216
DEAE filtrate	270	32	640,000	107	2.50$\bar{M}$	98	290
0 - 0.45 sat. $(NH_4)_2SO_4$ ppt.	50	11.0	195,000	33	0.13$\bar{M}$	5	-
0.45 - 0.65 sat. $(NH_4)_2SO_4$ ppt.	50	14.7	11,000	2	0.85$\bar{M}$	34	1,150
Extract of pptd starch filtrate	57	7.8	4,300	0.6	1.31$\bar{M}$	52	3,000
Hydroxylapatite, peak C	47.5	1.45	-	-	0.84$\bar{M}$	33	12,100

TABLE II

Properties of Bacillus Neutral Proteases

	B. subtilis NRRL B3411	B. subtilis var. Amylosacchariticus	Thermolysin
Molecular weight, literature	44,700	33,800	37,500
Molecular weight, found	35,100	40,500	-
ϵ, literature	13.6	13.8	17.6
ϵ, found	12.1	12.5	15.2
Zinc $\mu g/g$	1,364	1,450	1,450
Protease (Anson method (11))			
u/g, pH 7, casein	18.0×10^6 *	12.8×10^6	13.3×10^6
u/g, pH 7, hemoglobin	2.7×10^5	1.9×10^5	2.4×10^5
FAGLA Assay $10^2 k/E$, sec^{-1} mg/ml^{-1}	6.7	5.1	11.5
FAGLA/casein $(10^2 k/E)/(10^{-6}/g)$	0.37	0.41	0.86
Protease (McConn method (5))			
u/g, pH 7.3, casein, literature	13.6×10^6	12.6×10^6	-
u/g, pH 7.3, casein, found	11.0×10^6 **	9.7×10^6	12.6×10^6
% activity after 30 mins., 70°C	0	0	86

* assay on enzyme immediately after isolation
** assayed after 4 months storage at -5°C

ing a broad pH optimum (6.0 to 9.0) for activity, whereas the other two proteases have a sharp pH optimum at pH 7.3 for activity.

This differentiation is clearly marked in the hydroxylapatite (re)chromatography of the enzymes (fig. 2). Thermolysin is not adsorbed by hydroxylapatite, 96% of the enzyme activity being found in Peak A, whereas the NRRL B3411 and amylosacchariticus enzymes are adsorbed, Peak B containing 96 and 86% of the activity in each case, no activity being found in either A peak.

Neither the NRRL B3411 or amylosacchariticus proteases crossreacted with anti-thermolysin serum on Ouchterlony plates, nor did thermolysin crossreact

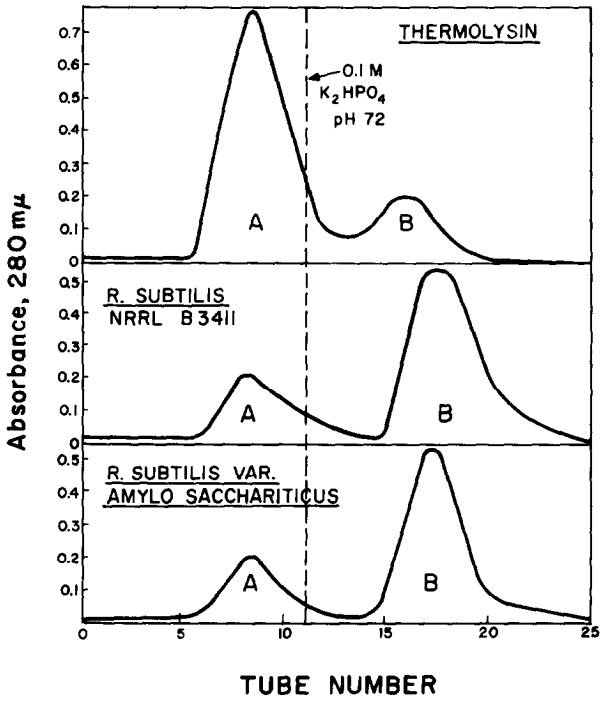

FIG. 2 Hydroxylapatite Chromatography of Neutral Proteases. 10 mg enzyme in 5 ml 0.1% $Ca(OAc)_2$ applied to a 1.4 x 7.5 cm column, 3 ml fractions collected at 5° and pooled peak fractions assayed for protease by the FAGLA method.

with anti-amylosaccharitícus protease serum. But the NRRL B3411 crossreacted with the amylosaccharitícus antiserum, the precipitation pattern indicating that the two enzymes were immunologically indistinguishable.

The quantitative immunological identity of the NRRL B3411 and amylosaccharitícus enzymes is shown in the parallel precipitation of protein and loss of enzyme activity with increasing addition of anti-amylosaccharitícus serum (fig. 3) and the equivalence points of the two enzymes (fig. 4).

Clearly thermolysin is quite distinct from the other enzymes. In the case of the alkaline proteases the immunological difference between the two types of enzymes was confirmed by the differences in amino acid composition between enzymes of the two groups (3), and a similar result was observed with

TABLE III

The Amino Acid Composition of Neutral Proteases

(Based on Molecular Weight of 36,400; 325 Residues)

	B. subtilis NRRL B3411		*B. subtilis* var. amylosaccharitícus	Thermolysin
	Found	Calculated from data of McConn (5)	Found	Calculated from data of Ohta (8)
Lys	17	16	17	12
His	5	5	5	9
Arg	8	8	9	10
Asp	48	48	49	44
Thr	29	29	31	24
Ser	31	33	32	24
Glu	27	26	26	21
Pro	9	11	12	8
Gly	30	29	30	37
Ala	28	27	28	29
Cys	0	0	0	0
Val	19	19	17	25
Met	4	4	2	2
Ile	13	14	13	19
Leu	21	20	23	17
Tyr	22	23	24	30
Phe	11	11	10	10
Try	3	3	3	5

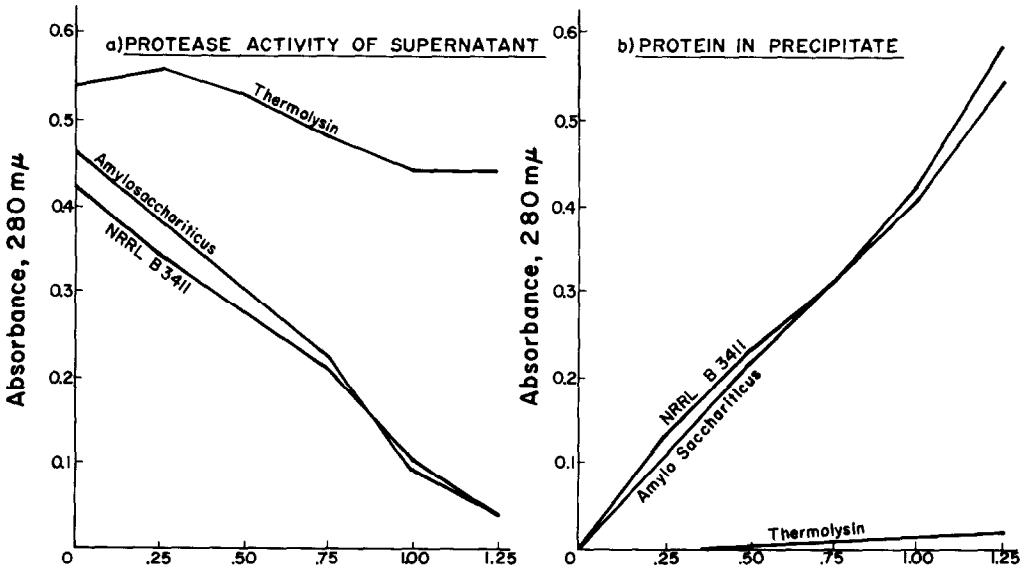

FIG. 3 Quantitave Precipitation of Protein and Enzyme Activity of Neutral Proteases by Anti-amylosaccharitiicus Serum.
0.5 mg enzyme in 0.25 ml physiological saline, antiserum and/or physiological saline to give a total volume of 1.5 ml. After 30 minutes at 37° and overnight at 5°, precipitate removed, washed, dissolved in 2 ml 0.1N NaOH for protein determination. Serum supernatant was diluted for assay by the casein digestion method.

the neutral proteases (table III). The composition differences between NRRL B3411 and amylosaccharitiicus enzymes are probably insignificant when the problems of autolysis and hence contamination are considered.

It is clear from this evidence that the B. subtilis NRRL B3411 and var. amylosaccharitiicus neutral proteases are indistinguishable by these methods and until more convincing evidence is presented it must be concluded that there are only two types of neutral proteases produced by Bacilli, namely thermolysin and the B. subtilis NRRL B3411 type enzyme. Apparently the mutation to produce β-amylase instead of α-amylase does not involve any change in the type of alkaline or neutral protease produced by the organism.

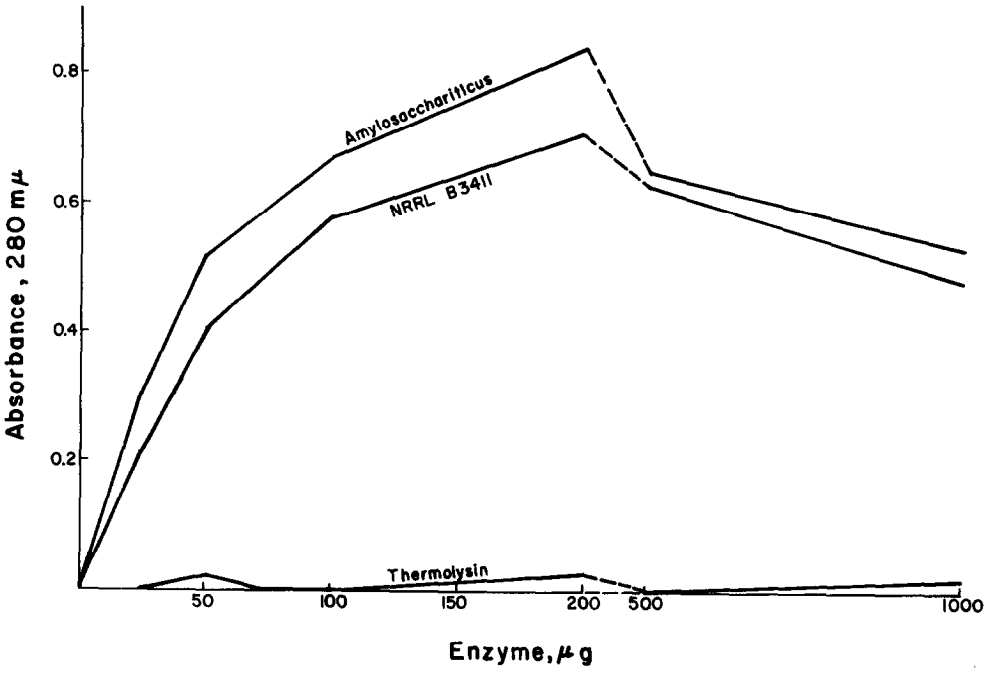

FIG. 4 Determination of the Equivalence Points of Neutral Proteases to Anti-amylosaccharilicus Serum.
Enzyme in physiological saline (0.1 or 1.0 mg/ml) was added to 0.5 ml antiserum and the mixture diluted to 1.5 ml with physiological saline. After 30 minutes at 37° and overnight at 5°, the precipitate was collected by centrifugation, washed with physiological saline and dissolved in 2 ml 0.1N NaOH for protein determination.

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