PROTEOLYTIC ENZYMES OF THERMOPHILIC BACTERIA—PART II

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Thermophilic bacteria are fairly widely spread and their proteolytic properties are called into play in several biochemical processes of importance in soil and agricultural chemistry and in the industry. These bacteria are capable of growing at unusually high temperatures. In Part I of this paper* attention has been drawn to the varied activities of these bacteria. A study of the proteolytic enzymes of these bacteria should prove useful. Besides the subject of microbial proteolytic enzymes has, as a whole, not been sufficiently investigated and further work with different types of micro-organisms and with better and more reliable experimental and analytical methods is necessary.

The present study is concerned with the proteinases of the three typical thermophilic bacteria—*Bacillus thermophilus, B. aerothermophilus* and *B. thermoacidurans,* cultures of which were obtained from the Lister Institute, London. Analytical and experimental methods here followed were, in general, similar to those described in Part I of this paper.

The endo- or exocellular nature of the proteinases of the thermophilic bacteria

A certain amount of controversy has centred round the question of the endocellular or exocellular nature of bacterial proteinases and in certain cases conflicting views have been expressed even with the same species of bacteria. Some confusion seems to have been caused by the failure to adequately distinguish between and separately determine the factors of multiplication, death of cells followed by autolysis and enzyme secretion by living cells. Virtanen and Tarnanen (1931) have stated that *Bacillus fluorescens* secretes a proteinase whose exocellular nature is assumed from the fact that the proteinase is found almost exclusively in the cell-free liquid. In a subsequent paper (1932) these authors stated that contrary to the general view on the subject, bacterial proteinases are exocellular in nature because filtrates from even young cultures of *Pseudomonas fluorescens* and *B. subtilis,* for instance, show proteolytic activity. Virtanen and Suolahti (1937) have

stated that contrary to Gorbach and Pirch (1936), proteinase of *B. fluorescens* liqu. is exocellular. Nevertheless Gorbach and Pirch (1937) have again observed that this proteinase is found in cultures only as a result of autolysis of dead cells. Maschmann (1938) has stated that *B. perfrigens* produces two proteinases, one of them being endocellular, and that the thiol activatable proteinase of the gas gangrene organism *Clostridium hystoliticum* is endocellular. On the other hand, Weil and Kocholaty (1937) and Kocholaty, Weil and Smith (1938) state that the thiol activatable proteinase of *Clostridium hystoliticum* is exocellular because the presence of the enzyme can be detected in filtrates from quite young cultures of this organism. Thus it would appear that conflicting conclusions have been reported even for the same species. It may be pointed out, however, that the mere fact that cultures are young is no guarantee that autolysis has not taken place.

In order to determine the endocellular or exocellular nature of the proteinases of thermophilic bacteria duplicate nutrient broth flasks were inoculated with each of the three bacteria and the proteinase content of the whole cultures, filtrates and washed cells was examined every six hours. The cells were obtained from the cultures by centrifuging at 3000 r.p.m. for thirty minutes and were re-suspended in distilled water so as to give the same volume as the amount of cultures they were reaped from. After thorough shaking 2 ml. were withdrawn for every estimation. Similarly 2 ml. of cell-free filtrates and 2 ml. of thoroughly shaken whole cultures were used for estimating the proteinase content of cell-free liquid and the total amount of proteinase in the culture respectively.

It is therefore obvious that the proteinase content of the whole cultures is always ahead of that of the filtrates. As the incubation time increases this difference becomes less and less. In the initial stages when the proteinase was just detectable in the whole cultures, the filtrates did not contain any and the cells accounted for all the proteolytic activity. Subsequently the difference between the proteolytic activity of the whole cultures and the filtrates roughly corresponded to the proteinase content of the cells. As the age of a culture increases the difference between the proteolytic activity of the whole culture and that of the cell-free filtrates becomes less and less and this progressive equalization corresponds to a decrease in the proteinase content of the cells. Thus the proteinase first appears in the cells and after some time it passes out into the filtrates. The decision whether the proteinases are really exocellular or endocellular is however dependent on the question whether when they diffuse out of the cell bodies into the surround-

TABLE I

Endocellular or Exocellular Nature of the Proteinases

Age of culture in hours	Whole culture		Cell-free filtrate		Suspension of cells	
	% Fall in initial Viscosity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
			B. thermophilus			
6 ..	0·00	0·00	0·00	0·00	0·00	0·00
12 ..	2·12	0·00	0·00	0·00	2·05	0·00
18 ..	8·30	2·37	0·00	0·00	7·80	1·89
24 ..	33·90	5·82	4·80	0·60	26·50	4·83
30 ..	57·80	10·53	29·15	2·13	28·55	7·81
36 ..	61·85	11·15	48·10	6·01	..	4·65
42 ..	60·05	11·01	50·35	9·00	10·15	2·11
47 ..	59·80	11·13	52·70	9·35	6·00	1·63
			B. aerothermophilus			
6 ..	0·00	0·00	0·00	0·00	0·00	0·00
12 ..	2·81	0·00	0·00	0·00	2·10	0·00
18 ..	6·65	1·12	0·05	0·00	6·50	1·09
24 ..	28·10	4·17	2·15	0·02	26·10	4·08
30 ..	49·50	8·73	23·50	3·13	26·35	5·91
36 ..	53·35	9·15	40·10	6·17	14·50	3·02
42 ..	56·00	9·97	53·70	7·83	3·20	2·11
			B. thermoacidurans			
6 ..	0·00	0·00	0·00	0·00	0·00	0·00
12 ..	0·00	0·00	0·00	0·00	0·05	0·00
18 ..	4·00	0·00	0·00	0·00	3·85	0·05
24 ..	29·95	4·37	12·05	1·15	17·30	3·37
30 ..	55·10	8·41	37·10	4·39	18·15	4·20
36 ..	55·60	9·17	43·50	6·16	..	3·15
42 ..	55·90	9·93	47·15	8·23	8·30	1·80
47 ..	54·05	9·83	48·10	8·25	6·05	1·45

ing medium the cells concerned are dead or alive. For ascertaining this point the course of proteinase increase or decrease in the whole cultures, in filtrates and in autolysed cells was re-determined and correlated with the estimation of total living and dead cells per ml. of the cultures.

Nutrient broth media was inoculated in each case from agar slant cultures and test samples were taken out aseptically after stated periods of incubation. After dilution smears were prepared, stained with carbol fuchsin and the total number of cells were counted. Simultaneously suitable decimal dilutions were plated on nutrient agar and counted after incubation for 48 hours. Estimation of total count by using the hæmocytometer, after rendering the diluted culture semisolid with agar so as to restrict bacterial movement, was found to be time consuming and inaccurate.

TABLE II

Hours after inoculation	Total No. of cells, 10^6/ml.	Viable cells, 10^3/ml.	Dead cells, 10^3/ml.	Whole culture: % Fall in initial viscosity in 30 min.	Whole culture: Increase in α-amino nitrogen, mgm./10 ml. in 48 hours	Filtrate: % Fall in initial viscosity in 30 min.	Filtrate: Increase in α-amino nitrogen, mgm./10 ml. in 48 hours	Cell suspension: % Fall in initial viscosity in 30 min.	Cell suspension: Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
				B. thermophilus					
4 ..	150	100	50	0·00	0·00	0·00	0·00	0·00	0·00
8 ..	..	..	..	0·00	0·00	0·00	0·00	0·00	0·00
12 ..	1,600	1,300	300	0·00	0·00	0·00	0·00	0·00	0·00
16 ..	10,500	7,000	3,500	1·25	0·00	0·35	0·00	0·80	0·00
20 ..	20,000	16,000	4,000	3·75	0·98	0·55	0·25	3·27	0·69
24 ..	30,000	15,000	15,000	42·70	6·72	24·10	3·97	20·15	3·19
				B. aerothermophilus					
4 ..	50	50	0	0·00	0·00	0·00	0·00	0·00	0·00
8 ..	700	..	..	0·00	0·00	0·00	0·00	0·00	0·00
12 ..	4,000	3,500	500	1·50	0·00	0·00	0·00	1·60	0·27
16 ..	15,000	10,000	5,000	3·70	0·78	0·30	0·15	3·55	0·65
20 ..	29,000	17,000	12 000	5·95	2·15	2·15	1·03	4·10	1·11
24 ..	32,000	17,000	15.000	39·40	7·32	20·05	4·05	20·90	3·79
				B. thermoacidurans					
4 ..	150	100	50	0·00	0·00	0·00	0·00	0·00	0·00
8 ..	600	350	250	0·05	0·00	0·00	0·00	0·00	0·00
12 ..	3,300	2,900	400	1·30	0·52	0·00	0·00	1·15	0·39
16 ..	29,000	23,000	6,000	4·85	2·25	0·75	0·10	4·15	2·07
20 ..	35,000	25,000	10,000	14·90	4·77	2·90	0·79	11·75	4·00
24 ..	39,000	18,000	21,000	38·75	8·15	19·80	3·07	22·80	4·98

An examination of the above data reveals that:—

1. death of the cells as determined by failure to multiply starts quite early after inoculation, in fact multiplication and death may be said to begin almost simultaneously; it is therefore inaccurate to say that in young cultures autolysis does not take place,

2. in case of the thermophilic bacteria little proteinase seems to be formed during the major part of the logarithmic phase of growth although the population of both dead and living cells is quite large,

3. cells reaped from thermophilic bacteria cultures which are yet in the logarithmic phase of growth also show little proteinase activity,

4. as soon as the logarithmic phase of growth is nearing completion in the cultures, proteinase can be detected in appreciable amount and then it increases rapidly both in the cells and in the surrounding liquid, the whole cultures still containing larger amounts than the cell-free filtrates and the proteinase contained in the cells themselves accounting for the difference and

5. the proteinase content of the whole cultures runs approximately parallel to the total cell count, whereas the proteinase content of the cell-free filtrates runs roughly proportional to the content of dead cells.

Unfortunately, these experiments had to be stopped after twenty-four hours, because after this the cells begin to show tendency towards formation of surface films which vitiate all cell counts. But it may be concluded that with the thermophiles the proteinase is generated within the cells after the maximum rate of multiplication has passed and that in all cases the proteinase is endocellular and appears into the surrounding liquid only after the cells have died.

Effect of calcium and magnesium on proteinase formation by the thermophilic bacteria and on the activity of the proteinase

Guillaumie (1935) has found that calcium activates trypsin. Merril and Clark (1928) found that in synthetic media certain bacteria produced a proteinase only in the presence of calcium and magnesium salts, although good growth was obtained even in their absence. Haines (1932) using *Pseudomonas* species and *Proteus* species has come to the conclusion that magnesium stimulates the growth of cells and calcium stimulates production of proteinase by the cells. These views on bacterial proteinases in general may have to be modified a little in view of the following findings with the thermophilic bacteria.

The procedure here adopted in studying the effect of a metal was to add a measured volume of the neutralised salt solution to the enzyme solution in such an amount that the desired final concentration would be obtained. The mixture was incubated at 30° C. for half an hour and its proteolytic activity was then measured in the usual way. Control experiments were performed in which the salt solution was replaced by an equal volume of distilled water.

TABLE III

Effect of Magnesium Salts—$MgSO_4 \cdot 7\ H_2O$

Source of Proteinase	Concentration of the salt in the enzyme solution	Initial rate, % Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
B. thermophilus ..	None	7·38	50·60	6·97
	M/2000	7·42	..	7·49
	M/1000	7·73	52·15	7·85
	M/250	7·80	53·40	8·37
B. aerothermophilus ..	None	6·88	45·75	6·11
	M/2000	6·92	45·92	6·38
	M/1000	7·05	46·15	6·69
	M/250	6·90	45·80	7·31
B. thermoacidurans ..	None	7·13	48·35	6·71
	M/2000	7·21	49·00	6·95
	M/1000	7·37	49·30	7·31
	M/250	7·40	49·55	7·78

TABLE IV

Effect of Calcium Salts—$CaCl_2$

Source of Proteinase	Concentration of the salt in the enzyme solution	Initial rate, % Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
B. thermophilus ..	None	7·28	50·05	6·91
	M/500	7·35	51·00	7·43
	M/250	7·35	51·30	7·95
B. aerothermophilus ..	None	6·85	45·15	6·05
	M/500	6·95	45·40	6·78
	M/250	6·96	45·90	7·17
B. thermoacidurans ..	None	7·05	47·60	6·59
	M/500	7·15	47·55	7·01
	M/250	7·20	47·90	7·48

The activating effect of both calcium and magnesium ions on the amount of α-amino acids liberated is moderate but definite and regular. The conclusion of Haines that presence of calcium salts increases the amount of proteinases produced may, however, be due simply to the conversion of the proteinase already present into a more active form. Haines did not compare the proteolytic acitivity of filtrates from cultures prepared in media containing calcium salts with filtrates from cultures prepared in ordinary media but after adding calcium salts a little while before testing for the proteolytic activity. With the thermophilic bacteria the proteolytic activity of filtrates obtained from cultures in broth containing calcium and magnesium salts was higher than that of filtrates obtained from cultures in plain broth but was nearly the same as that of plain broth culture filtrates which had been subsequently activated by the addition of calcium and magnesium salts. This is apparent from Table V. With Merril and Clark's synthetic medium it was found

TABLE V

A—Filtrates from plain broth cultures.

B—Filtrates from cultures made in broth containing calcium and magnesium salts.

C—Filtrates from plain broth cultures, but with calcium and magnesium salts added subsequently.

Source of Proteinase	Nature of filtrate	Initial rate, % Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
B. thermophilus ..	A	7·35	50·30	5·67
	B	7·47	53·25	6·41
	C	7·42	53·90	6·37
B. aerothermophilus ..	A	6·83	47·35	5·01
	B	6·72	48·40	5·93
	C	6·87	48·75	5·90
B. thermoacidurans ..	A	6·31	50·80	5·63
	B	7·17	51·90	6·37
	C	7·11	51·95	6·38

that good growth could be obtained only when both calcium and magnesium salts were present, filtrates from such cultures had appreciable proteolytic activity although less than that of filtrates from broth cultures.

Effect of iron and copper on the proteinases of the thermophilic bacteria

Unlike papainases the proteinases of thermophilic bacteria are not suppressed by copper salts. The effect of ferrous and ferric salts is not marked except that at high concentrations of ferric ions a mild suppression of the activity of these proteinases is observed. The data about the effect of ferrous and ferric salts is, however, not reproduced here as the effect was inconsistent.

TABLE VI

Effect of Copper Salts —$CuSO_4 \cdot 5H_2O$

Source of Proteinase	Concentration of the salt in the enzyme solution	Initial rate % Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
B. thermophilus ...	None	6·95	52·10	6·17
	M/1000	6·87	51·85	6·10
	M/250	7·05	52·35	6·28
	M/100	7·10	52·50	6·32
B. aerothermophilus ..	None	6·35	46·80	5·63
	M/1000	6·13	45·45	5·51
	M/250	6·29	46·75	5·59
B. thermoacidurans ..	None	5·80	40·50	4·98
	M/1000	5·95	41·30	5·02
	M/250	5·60	39·95	4·91
	M/100	6·05	42·95	5·10

These proteinases are therefore quite different from papainases in their response towards copper ions.

Effect of oxidising and reducing agents on the activity of the proteinases of thermophilic bacteria

It is quite well known that apart from pH, the activity of enzyme solutions is also affected by the presence of oxidising and reducing agents. In case of the proteinases, a study of the effect of oxidising and reducing agents throws some light on the nature and mode of action of the enzymes, hence the importance of this study. The effect of a number of reagents has been tried on the proteinases of *B. thermophilus, B. aerothermophilus* and *B. thermoacidurans* and the results are summarised in Tables VII, VIII and IX. Although the effect of oxidising agents is on the whole that of suppression of the proteolytic activity, yet the irregular nature of the data and the limited extent of the effect do not allow of any definite conclusions to be

reached. Agreement between duplicate sets of data was generally poor. Uniform and regular suppression of the bacterial proteolytic activity was obtained only with methylene blue and iodine. The latter prevents proteolytic activity in all cases.

The enzyme solution and the solution of the reagent were mixed in requisite proportions and incubated at 30° C. for half an hour before mixing with the substrate. For blank experiments an equal volume of distilled water was substituted for the solution of the reagent. The solutions of different reagents were pre-adjusted to the pH of the enzyme solutions.

TABLE VII

Effect of Oxidising and Reducing Agents

Ragent and concentration	*B. thermophilus*		
	Initial rate, % Fall in initial viscosity in 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen mgm./10 ml. in 48 hours
Nil ..	7·39	52·60	6·61
Potassium bromate M/100 ..	6·87	52·10	6·43
Potassium iodate M/100 ..	7·10	49·50	6·43
Methylene blue M/250 ..	6·27	43·10	5·53
Hydrogen peroxide M/100 ..	7·17	49·55	6·50
Potassium ferrocyanide M/200 ..	7·30	53·19	6·55
Potassium ferricyanide M/200 ..	7·11	50·00	6·47
Iodine M/350 ..	Nil	Nil	Nil

TABLE VIII

Effect of Oxidising and Reducing Agents

Ragent and concentration	*B. aerothermophilus*		
	Initial rate, % Fall in initial viscosity in 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen mgm./10 ml. in 48 hours
Nil ..	7·03	49·15	6·27
Potassium bromate M/100 ..	6·89	47·10	6·02
Potassium iodate M/100 ..	6·81	47·15	6·13
Methylene blue M/250 ..	5·97	42·00	5·43
Hydrogen peroxide M/100 ..	6·87	47·30	6·21
Potassium ferrocyanide M/200 ..	7·01	50·10	6·05
Potassium ferricyanide M/200 ..	6·83	46·90	6·21
Iodine M/350 ..	Nil	Nil	Nil

TABLE IX

Effect of Oxidising and Reducing Agents

Ragent and concentration	*B. thermoacidurans*		
	Initial rate, % Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
Nil ..	6·95	49·90	6·07
Potassium bromate M/100 ..	6·87	49·00	6·02
Potassium iodate M/100 ..	6·89	48·15	6·11
Methylene blue M/250 ..	6·01	43·95	5·17
Hydrogen peroxide M/100 ..	6·87	48·05	6·00
Potassium ferrocyanide M/200 ..	6·97	51·10	6·05
Potassium ferricyanide M/200 ..	6·90	48·95	6·10
Iodine M/350 ..	Nil	Nil	Nil

Effect of peptone on the hydrolysis of gelatin by the proteinases of thermophilic bacteria

A concentrated solution of Witte's peptone was added to a 3% gelatin solution in McIlvaine's buffer at the optimum pH in such an amount that the final concentration of peptone would be 1%. Any alteration in pH incidental to the addition of peptone was adjusted. The addition of peptone increased the relative viscosity of the substrates by about 10%. But since fall of viscosity was being measured as per cent. of initial viscosity the error introduced was not considerable especially in view of the fact that the alteration in the rate of hydrolysis brought about by the addition of peptone was of a high magnitude. The enzyme solutions which were filtrates from not more than thirty-six hour old cultures showed little activity when tested against peptone alone. 2 ml. of the enzyme solution were used per 10 ml. of substrate.

TABLE X

Source of Proteinase	% Fall in initial viscosity in 30 minutes		Increase in α-amino nitrogen, mgm./10 ml.	
	Without Peptone	With peptone	Without Peptone	With Peptone
B. thermophilus ..	51·05	7·05	6·87	1·87
B. aerothermophilus ..	49·35	4·50	6·67	1·72
B. thermoacidurans ..	44·40	5·65	6·00	0·97

Thus peptone depressed the proteolytic activity of the bacterial proteinases.

Effect of peptone on proteolysis of gelatin substrates of different concentrations

It was now of interest to find out whether the effect of peptone is competitive or non-competitive with the substrate. Substrates containing 3·5, 2·7, 1·9 and 1·1% gelatin were mixed with Witte's peptone to give a final concentration of 1% peptone and any alteration in pH was adjusted. Duplicate sets were run without peptone.

TABLE XI

% Gelatin in substrate	% Fall in initial viscosity in 30 minutes		Increase in α-amino nitrogen, mgm./10 ml. in 48 hours	
	Without Peptone	With Peptone	Without Peptone	With Peptone
	Bacillus thermophilus			
3·5	40·10	20·35	6·11	3·47
2·7	40·00	10·50	5·15	2·03
1·9	48·15	7·10	4·02	0·95
1·1	52·23	3·00	3·83	0·35
	Bacillus aerothermophilus			
3·5	40·10	18·15	9·10	5·37
2·7	44·25	10·50	8·02	3·01
1·9	49·85	6·10	6·62	1·69
	Bacillus thermoacidurans			
3·5	43·15	22·10	7·38	4·25
2·7	46·65	8·05	6·32	2·91
1·9	48·10	4·90	5·43	1·70
1·1	51·30	2·10	4·10	0·95

It is therefore obvious that in every case the extent of depression in proteolytic activity caused by the presence of peptone decreases with increase of gelatin concentration in the substrate, *i.e.*, peptone depression is competitive with the substrate. It appears that although peptone itself is not hydrolysed by the enzyme which hydrolyses gelatin, yet it competes with the gelatin for attachment to the active group in the enzyme molecule, or at a group which is so near the active group of the enzyme molecule that through spatial hindrance the peptone molecule can prevent the attachment of the gelatin to the enzyme.

SUMMARY

1. The thermophilic bacteria *Bacillus thermophilus*, *B. aerothermophilus* and *B. thermoacidurans* produce very active proteinases which appear to be

endocellular in character and are found in culture filtrates only as a result of autolysis of the cells.

2. Filtrates obtained from broth cultures containing calcium and magnesium salts are proteolytically more powerful than those obtained from cultures prepared with ordinary broth, but the effect of these metals is on the activity of the proteinases and not on the quantity of the enzyme produced.

3. Unlike papainases the proteinases of the thermophilic bacteria are not affected by copper ions.

4. Activity of the proteinases of thermophilic bacteria is suppressed by methylene blue and is prevented by iodine.

5. Although peptone itself is not hydrolysed by the proteinases of thermophilic bacteria, yet it hinders the hydrolysis of gelatin. The evidence obtained tends to show that the effect of peptone is probably due to competition by peptone with the gelatin for attachment to the active centre of the enzyme molecule, or a centre very close to the active centre.

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