PROTEOLYTIC ENZYMES OF THERMOPHILIC BACTERIA—PART I

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Received November 19, 1945

THERMOPHILIC bacteria are widely distributed in soil, etc., and are responsible for a large number of biological processes which are of importance in nature, in agriculture and in certain industries. Proteolytic breakdown is an essential feature of many of these processes. These bacteria can grow at high temperatures often close to the coagulation temperature of their albumins. In Beccari system of garbage fermentation, for example, temperatures above 50° C. are reached and thermophilic bacteria undoubtedly play a part liquefying the proteins in the initial stages and decomposing amino acids in the later stages. de Kruyff (1910) and Bergey (1919) have isolated from soil, manure, dust, etc., several species of thermophilic bacteria, many of which were of a proteolytic nature. Rabinowitsch (1895) has found thermophiles in soil and Weinzirl (1919) found *B. ærothermophilus* in canned string beans. Pricket (1928) has found *B. ærothermophilus* wide spread in hay, dust, beef extract, water, milk and agar. Thermogenic fermentation in the production of silage, processing of tobacco, cacoa and coffee as well as the harmful heating up of hay is well known and according to James *et al.* (1928) nitrogen metabolism and thermogenesis are interrelated. According to Dunez (1933) nitrogen in the form of albumin and its degradation products plays an important role in the fermentation of manure piles by thermophilic bacteria. Damon and Feirer (1925) have found aerobic and proteolytic thermophils in horse manure. Hops and oil-seeds often heat up on account of the activity of thermophilic bacteria. Microbial proteolytic thermogenesis of wool has been studied by Barker (1929). It is well known that thermophilic bacteria and molds are the active agents in manure fermentation. Damage caused to canned meat, fish, etc., by thermophilic bacteria is well known and Rokusho and Fukutome (1937) ascribed a type of spoilage of soya beans to the proteolytic activity of thermophilic bacteria.

The nature and properties of microbial proteinases and their mode of formation is still imperfectly understood. Considerable amount of work is still necessary with different types of micro-organisms and by using more reliable

and more decisive methods of analysis and experimental methods. Proteolytic enzymes of thermophilic bacteria do not seem to have been investigated at all. These bacteria thrive at unusually high temperatures and this fact, coupled with their practical importance, renders them an intriguing subject for study even apart from any theoretical interest in the nature of microbial proteinases in general. The present investigations are devoted to a study of the proteinases of three typical thermophilic bacteria:—

Bacillus thermophilus

Bacillus aerothermophilus

Bacillus thermoacidurans.

Cultures of these organisms were obtained from the Lister Institute, London.

Methods of Analysis

The methods of analysis employed by a majority of the previous workers in the study of microbial proteinases do not seem to have been quite satisfactory. For instance, a large number of investigators have estimated the amount of α-amino acids liberated by the proteinase without having previously verified the absence of poly- and dipeptidases in their enzyme extracts. Besides estimation of liberated amino acids alone does not throw any light on the initial stages of proteolytic breakdown when the protein undergoes profound physical changes without any appreciable increase in α-amino nitrogen. It would be more satisfactory to use a scheme of analysis whereby both initial and final stages of proteolytic action can be determined. The analytical schemes for various proteins actually adopted in the present study comprise firstly the determination of the initial stage of proteolysis, secondly the final stage, *i.e.*, when hydrolysis has ceased or has become very slow an in addition the determination at stages intermediate between the intitial and final stages.

The most convenient method for studying the initial stages must necessarily depend upon the physico-chemical and colloidal properties of the protein. In the present investigations a sensitive method for gelatin solutions was afforded by the measurement, in an Ostwald viscometer, of the progressive fall in viscosity of the solution in the presence of a proteinase. Viscosity/time curves were plotted and when extrapolated to zero time they gave the relative initial speed of hydrolysis which is a good measure of the very first stages of proteolysis of gelatin. Whenever parallel experiments were to be conducted at different hydrogen-ion concentrations then due corrections were made for the effect of pH on the specific viscosity of gelatin solutions. Corrections due to any effect of buffer, heating, etc., can be

ignored as these conditions are always reproducible. In addition to determining the initial speed of hydrolysis the per cent. fall in initial viscosity in a definite period of time (30 minutes) was also calculated from the same set of observations and this gave an idea of the progress of proteolysis.

With the albumins the initial stages of hydrolysis were followed by precipitating the unaltered protein by boiling the solution after bringing it to the isoelectric point of the protein. A small quantity of pure sodium chloride was added to the solution before boiling in order to increase the range of maximum coagulability of the unaltered albumin to some extent on both sides of the isoelectric point, thus avoiding the extra time and labour necessary to bring the solution exactly to the isoelectric point of the albumin. The intermediate stage of proteolytic hydrolysis was determined by precipitation with trichloracetic acid.

The final stage of proteolytic breakdown in case of all proteins was studied by estimating the increase of free α-amino acids by using the micro Van Slyke method, due corrections being made for the auto-hydrolysis of the proteinase solution.

Proteinase Solutions

The bacteria were cultivated in nutrient broth by incubation at 50° C. for thirty-six to forty-eight hours. The cultures were centrifuged and filtered through Chamberland candles yielding cell free filtrates which were proteolytically very active and were used directly.

Substrates

Previous workers have generally used only one kind of protein in their investigations on microbial proteinases. The results obtained by them therefore do not appear to be based on very firm foundations. 'Bacto' gelatin flakes, Merck's dried egg albumin and casein (Hammersten) were the three proteins employed in the present study. In addition some experiments were made with serum albumin and with denatured albumin prepared in the laboratory. Egg albumin solutions were dialysed in parchment bags in ice cold distilled water before mixing with the buffer solution in order to remove the lower degradation products which accompanied the true albumin. Previous investigators do not seem to have taken this precaution. McIvaine's citrate-phophate mixtures were used as buffer as they do not hinder proteolysis, compare Weil and Kocholaty (1937).

Experimental Methods

Several stoppered test-tubes containing 10, 20 or 30 ml. of the protein solution of the required concentration and pH were prepared with the buffer

and 1% phenol or a drop of toluene per tube. These were brought to 38–39° C. in an incubator before starting an experiment. A test-tube was taken out. 1 ml. or 2. ml. of the enzyme solution was run in and the test-tube was shaken. In case of experiments with gelatin substrate a stop watch was started simultaneously with the shaking and 2 ml. of the mixture was pipetted out immediately into a dry Ostwald type of viscometer previously maintained at 37° C. in a thermostat. Viscometric readings were started using a second stop watch five minutes after the mixing of the enzyme solution and the substrate. During this time thermal equilibrium had already been attained in the viscometer. Readings were repeated every five minutes for about fifty minutes after which the fall in viscosity was very slow. Initial speed of fall of viscosity was calculated by extrapolating viscosity/time curves to zero time and also the per cent. fall in initial viscosity in 30 minutes was calculated. Finally α-amino nitrogen was estimated in 2 ml. of the mixture, using a micro Van Slyke apparatus, immediately after completion of the viscosity readings and again after incubating at 40° C. for a definite period. There is no measurable increase in α-amino nitrogen during the one hour or so which must elapse after mixing of the enzyme solution with the substrate and before an aliquot portion can be analysed in the Van Slyke apparatus.

When the substrate protein used was an albumin, then aliquot volumes were pipetted out immediately after mixing the substrate solution with the proteinase solution and analysed for (*a*) the amount of organic nitrogenous compounds not precipitated by boiling at the isoelectric point of the protein, (*b*) the amount not precipitated by mixing with an equal volume of 8% tri-chloracetic acid and (*c*) the free α-amino acids by estimation by the Van Slyke method. The three estimations were repeated after incubation at 40° C. for a definite length of time. Micro-Kjeldahl method was used for the estimation of nitrogen compounds.

pH Optima of the hydrolysis of gelatin by the proteinases of thermophilic bacteria

2 ml. each of the proteinase solution were mixed with 20 ml. each of 3% 'Bacto' gelatin solutions prepared at different pH in McIlvaine's citrate-phosphate buffer and viscometric determinations and estimation of increase in α-amino acids were carried out as described above. The results are summarised in Tables I, II and III below.

All the three proteinases therefore have their pH optima in the alkaline region, *e.g.*, at pH 8·1 to 8·3 for *Bacillus thermophilus*, pH 7·5 to 7·7 for *B. aerothermophilus* and pH 8·0 to 8·1 for *B. thermoacidurans*. In this respect, therefore, they resemble the tryptases.

TABLE I

Optimum pH of the proteinase of B. thermophilus

pH	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
3·0	0·67	5·35	0·00
4·0	0·94	10·80	0·00
5·0	2·24	21·30	3·10
6·0	5·37	40·15	6·13
7·0	8·15	48·00	8·65
7·5	9·65	51·35	10·13
8·0	10·63	55·50	11·67
8·5	10·50	54·60	11·68
9·0	10·27	51·25	10·15
9·5	10·00	51·00	9·40
10·0	7·28	46·50	8·15

TABLE II

Optimum pH of the proteinase of B. ærothermophilus

pH	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
3·0	1·85	2·25	0·00
4·0	3·10	4·10	1·15
5·0	5·65	23·75	3·65
6·0	7·55	45·10	6·41
7·0	9·15	55·90	8·98
7·5	12·10	59·15	9·53
8·0	11·50	56·45	9·00
8·5	10·00	55·40	8·75
9·0	7·55	49·15	7·15
9·5	4·25	44·00	6·37
10·0	3·40	32·50	4·66

TABLE III

Optimum pH of the proteinase of B. thermoacidurans

pH	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
3·0	1·68	1·95	0·00
4·0	2·15	3·00	0·00
5·0	3·17	19·65	3·15
6·0	5·68	32·70	5·01
7·0	7·86	44·00	6·65
7·5	9·79	48·35	8·22
8·0	11·53	50·65	8·68
8·5	9·61	49·30	8·25
9·0	9·38	47·45	8·00
9·5	7·95	44·65	6·41
10·0	5·40	30·40	4·78

Peptone splitting enzyme in filtrates from the cultures of the thermophilic bacteria

Besides the true proteinase the thermophilic bacteria also produce an enzyme which can hydrolyse peptone and which is distinct from the proteinase. The enzyme solutions used in these experiments were filtrates from four days' old cultures. 20 ml. of a 2% solution of Witte's peptone made up at different pH in McIlvaine's citrate-phosphate buffer were incubated at 40° C. for forty-eight hours with 2 ml. of the enzyme solution and the increase in α-amino acids was estimated by Van Slyke's method. The results are tabulated in Table IV and it is clear that the optimum pH of this enzyme is between 7·5 and 8·0.

TABLE IV

Optimum pH of bacterial peptonase

Increase in α-amino nitrogen mgm./10 ml. in 48 hours

pH	*B. thermophilus*	*B. aerothermophilus*	*B. thermoacidurans*
3	0·00	0·00	0·00
4	0·00	0·00	..
5	..	0·33	..
6	0·57	0·75	0·93
7	1·63	1·82	2·15
8	2·00	1·98	2·20
9	1·04	0·86	1·01
10	0·22	0·45	..

Course of formation of proteinase and peptonase in cultures of thermophilic bacteria

The course of increase or decrease of proteinase and of peptone splitting enzyme in cultures was followed daily during an incubation period of one week. As before culture filtrates served as the enzyme solutions. The results are summarised in Table V.

The polypeptidase capable of splitting peptone therefore first appears when the cultures are two or three days old and then goes on increasing steadily, whereas the proteinase is found in culture filtrates in appreciable quantity within twenty-four hours of inoculation and reaches a maximum in two or three days, after which the proteinase activity of the filtrates decreases gradually. Thus filtrates from thirty-six or forty-eight hours old cultures do not contain any enzyme capable of further hydrolysing the products obtained by proteolytic degradation of a true protein. Most previous investigators do not seem to have taken the precaution of ensuring that their enzyme extracts were free from peptonases and polypeptidases

TABLE V

Course of formation of proteinase and peptone splitting enzyme by bacteria

2 ml. of the culture filtrate to every 10 ml. of 3% 'Bacto' gelatin substrate or 10 ml. of 2% Witte's peptone substrate, both at optimum pH

Age of culture in hours	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*	
			Proteinase	Peptonase
		B. thermophilus		
24	4·63	22·15	4·00	0·10
48	12·25	61·70	13·73	0·35
72	12·90	62·50	13·80	0·80
96	11·86	57·65	11·37	1·13
120	10·75	54·10	10·73	1·72
144	8·12	47·25	8·33	1·96
160	6·67	33·40	6·78	2·17
		B. aerothermophilus		
25	4·79	30·10	4·39	0·00
49	11·30	35·60	10·73	0·15
74	11·35	56·75	11·17	0·40
97	10·17	53·15	9·63	1·12
121	9·00	47·10	8·17	1·68
145	7·17	40·25	6·83	2·05
162	6·90	31·70	5·91	2·30
		B. thermoacidurans		
24	4·71	28·10	4·33	0·00
48	9·73	47·85	8·13	0·05
72	9·96	49·30	8·62	0·23
96	8·89	44·10	8·00	1·03
120	7·73	37·35	7·63	1·61
144	6·15	30·00	7·11	1·93
160	5·33	21·30	6·97	2·18

although the methods of analysis adopted by them depended solely on the estimation of increase of free α-amino acids. Maschmann (1937) has tried to eliminate the polypeptidase from his enzyme solutions by a laborious system of adsorption.

Hydrolysis of casein by the proteinases of the thermophilic bacteria

The substrate solutions were prepared at different pH in McIlvaine's buffer and contained generally 1·4 to 3·0 mgm. organic nitrogen per ml. Typical results obtained with casein are summarised in Table VI from which it is clear that casein is efficiently hydrolysed, the optimum pH being 7·3 to 7·7.

TABLE VI

Hydrolysis of casein by bacterial proteinases

Proteinase from	Increase in α amino-nitrogen, mgm./10 ml. in 48 hours					
pH	..	5	6	7·5	8·5	9·5
B. thermophilus ..	..	3·01	3·43	5·21	4·83	3·37
B. aerothermophilus ..	..	5·35	7·08	9·10	6·05	..
B. thermoacidurans ..	..	1·42	1·53	4·24	3·11	2·56

Hydrolysis of albumins by bacterial proteinases

In contrast to casein and gelatin, egg albumin is very resistant to proteolytic action of the proteinases under study. This is obvious from the data summarised in Tables VII, VIII and IX. The optimum hydrogen ion concentration appears to be at pH 7·5 to 8·0 approximately.

TABLE VII

Hydrolysis of albumin: B. thermophilus

pH	Increase in nitrogen not precipitated at isoelectric point, mgm./40 ml. in 48 hours	Increase in nitrogen not precipitated in 4% trichloracetic acid, mg./40 ml. in 48 hours	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
3	0·00	0·00	0·00
4	0·00	0·00	0·00
5	0·00	0·00	0·00
6	0·00	0·00	0·00
7	6·32	2·35	0·50
8	8·41	2·58	0·68
9	3·27	1·25	0·00
10	0·00	0·00	0·00

TABLE VIII

Hydrolysis of albumin: B. ærothermophilus

pH	Increase in nitrogen not precipitated at isoelectric point, mgm./40 ml. in 48 hours	Increase in nitrogen not precipitated in 4% trichloracetic acid, mgm./40 ml. in 48 hours	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
3	0·00	0·00	
4	0·00	0·00	
5	0·00	0·00	
6	1·32	0·00	Nil
7	3·86	1·37	
8	4·20	1·43	
9	1·05	0·00	
10	0·00	0·00	

TABLE IX

Hydrolysis of albumin: B. thermoacidurans

pH	Increase in nitrogen not precipitated at isoelectric point. mgm./40 ml. in 48 hours	Increase in nitrogen not precipitated in 4% trichloracetic acid, mgm./40 ml. in 48 hours	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
3	0·00	0·00	
4	0·00	0·00	
5	0·00	0·00	
6	1·94	0·00	Nil
7	5·38	1·38	
8	5·93	2·11	
9	2·15	0·00	
10	0·00	0·0·	

Serum albumin and serum and the proteinases of thermophilic bacteria

Serum albumin was found to be even more resistant to hydrolysis by the proteinases of the thermophilic bacteria. Only very slight increase in organic nitrogen not precipitable by boiling at isoelectric point or by trichloracetic acid could be detected at all the hydrogen-ion concentrations studied. From this very slight increase it was impossible to find out the optimum pH of hydrolysis but it appeared to be somewhat above 7. Increase in α-amino nitrogen could not be detected. Data is not cited here as extent of hydrolysis was very meagre. Incidentally it was found that not merely serum albumin is resistant to attack itself but also that liquid serum strongly inhibits the activity of the proteinases here studied. As expected, denaturation of egg albumin and serum albumin rendered them more readily available for attack by these proteinases.

Relationship between time and percentage of hydrolysis

The bacterial proteinases studied here are fairly powerful and it was found that linear relationship was obtained by plotting per cent. fall in viscosity of gelatin solution against the square root of time only. This is illustrated for *Bacillus thermophilus* proteinase in Fig. 1. The curves in case of the proteinases of *B. aerothermophilus* and *B. thermoacidurans* are quite similar.

Relationship between percentage of hydrolysis and enzyme concentration

On the basis of the Michaelis and Menton's theory it would be expected that velocity of hydrolysis will vary directly with the enzyme concentration. Varying results have been obtained regarding relationship of percentage of

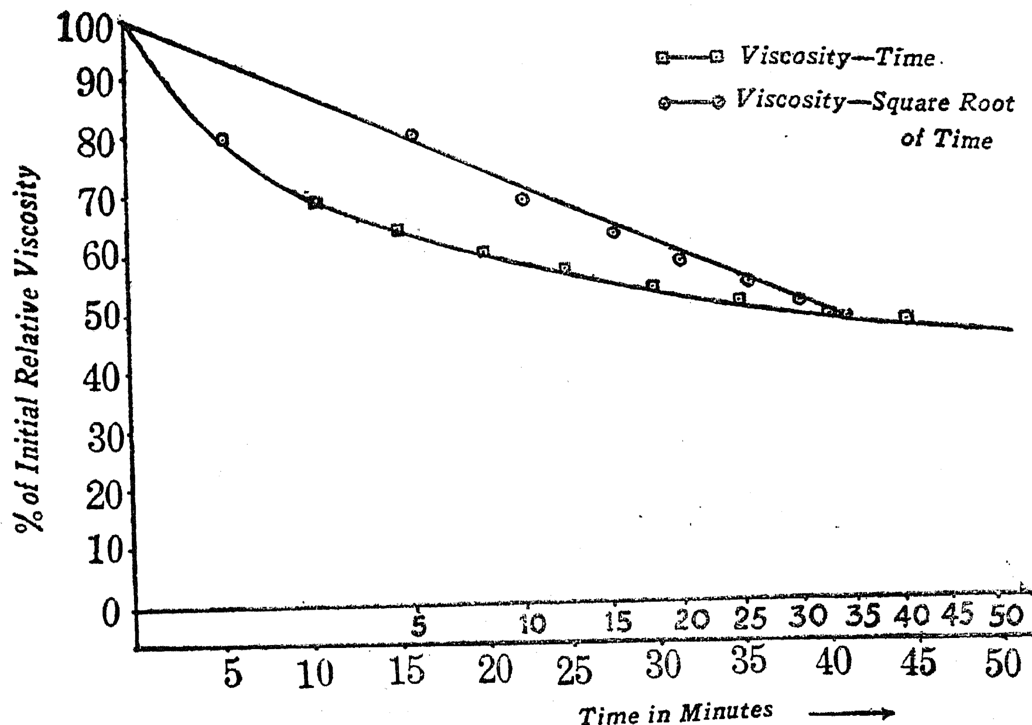

FIG. 1. Velocity/Time Curves for Proteinase of B. thermophilus

hydrolysis to enzyme concentration by different workers in case of trypsin. [See for example Bergmann and Pojarlieff (1934); Bergmann and Föhr (1933) and Farber and Wynne (1935).]

In case of pepsin, Anson and Mirsky (1932) have found direct relationship between the rate of hydrolysis and enzyme concentration only when enzyme and substrate concentrations were low. Direct relationship has been found for gelatin hydrolysis in the case of malt proteinases by Luer and Malsch (1929). In the case of proteinases of the thermophilic bacteria here studied linear relationship was obtained irrespective of whether the initial rate of hydrolysis or the progressive rate as represented by the per cent fall in initial viscosity of gelatin solutions in thirty minutes or measurement of the amount of free amino acids set free is used as the basis. This is shown graphically in Fig. 2.

In 1913 Michaelis and Menton announced that enzymic processes are preceded by a combination of the enzyme molecule and the substrate molecule. Evidence that has later on been accumulated by some other workers has generally supported this view. Kinetics of enzymic reactions can be satisfactorily interpreted on this basis. Further evidence in support of this view is afforded by the fact that many enzymes are protected by their substrates against heat. For example, the thermostability of the microbial enzymes here studied is greater in presence of proteins and peptone than in their

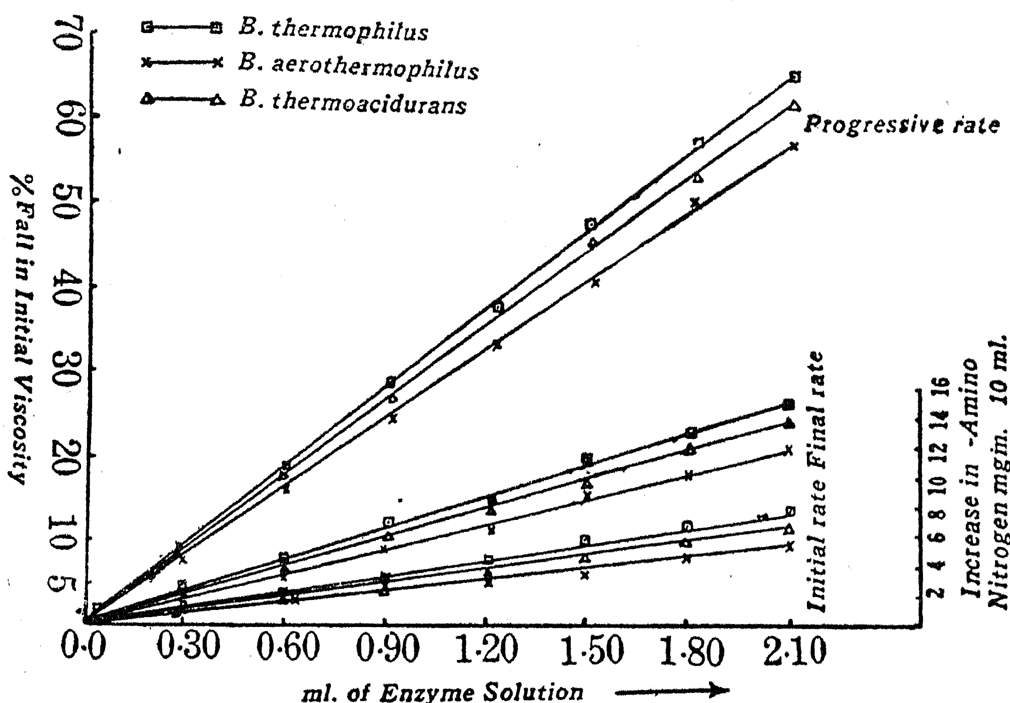

Fig. 2. Enzyme Concentration and Hydrolysis

absence and the destruction of activity of trypsin solution which takes place rapidly at room temperatures is prevented by the addition of a very small quantity of gelatin or peptone. Data need not be cited here, but the effects are quite striking. At least in one case, Stern (1936), formation of the intermediate enzyme-substrate complex has been proved by spectrum analysis. According to Northrop (1932), however, trypsin does not form a compound with its substrate. More recent work by Farber and Wynne (1935) has, on the other hand, proved that Michaelis and Menton's equation is in fact applicable.

In the present experiments same quantity of enzyme was added to gelatin substrates of different concentrations varying from 1·2 to 4%. The data are set forth in Table X.

TABLE X

Velocity of hydrolysis and substrate concentration

Proteinase of	% Fall in initial viscosity in 30 minutes at substrate concentration of				
	1·2%	1·9%	2·6%	3·3%	4%
B. thermophilus	..	26·20	33·35	37·60	44·15
B. aerothermophilus	18·75	26·95	33·10	35·00	39·05
B. thermoacidurans	16·30	15·80	21·20	20·50	..

On equations based on Michaelis and Menton's theory, Linweaver and Burk (1934) have shown that the plot of the ratio of initial substrate concentration to reaction rate (S/V) against the initial substrate concentration (S) yields a straight line for the case in which an enzyme-substrate compound consisting of one molecule each of the enzyme and the substrate is postulated. This method of plotting has also been employed by Greenberg and Winnick (1940) in case of certain plant proteases.

In the case of microbial proteinases here studied S/V plotted against S gave straight line curves where V was equal to per cent fall in initial viscosity in first five minutes or first thirty minutes. This is evident from Fig. 3.

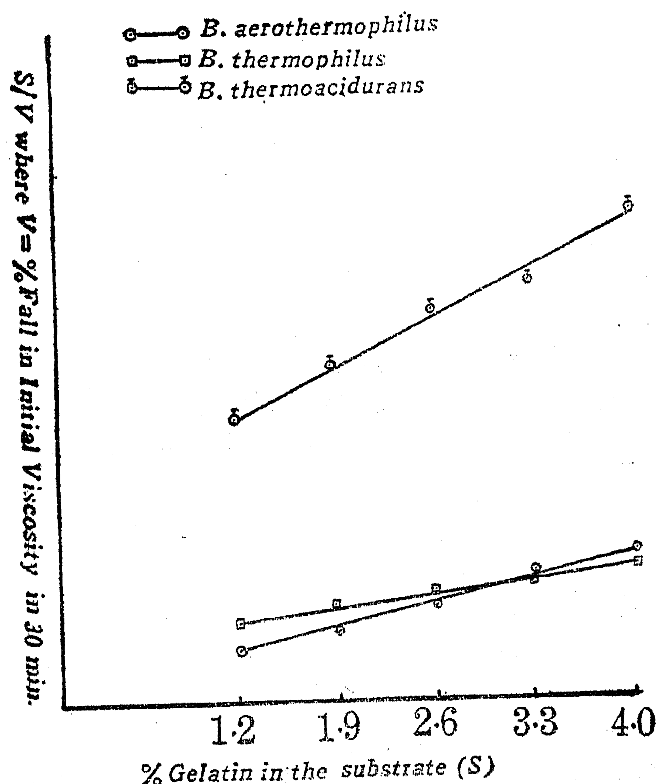

FIG. 3. Substrate concentration against ratio of Substrate Concentration and Viscosity

From these results it appears that with these bacterial proteinases the enzyme combines with the substrate before hydrolysing it and that the enzyme-substrate complex consists of one molecule each of the enzyme and the substrate.

SUMMARY

1. The thermophilic bacteria ***Bacillus thermophilus, B. aerothermophilus*** and *B.* ***thermoacidurans*** produce powerful proteinases which can be detected in the culture filtrates.

2. These proteinases resemble tryptases in their optimum hydrogen-ion requirements and hydrolyse gelatin and casein readily and albumins sparingly, unless the albumin has been previously denatured.

3. In addition to the proteinases these thermophilic bacteria also produce a polypeptidase capable of hydrolysing peptone, but this enzyme appears in culture filtrates much later than the proteinases.

4. Velocity of gelatin hydrolysis by these proteinases varies directly as the square root of time and percentage of hydrolysis varies directly with the enzyme concentration.

5. Relationship of substrate concentration and rate of hydrolysis shows that in case of gelatin Michaelis and Menton's equation is applicable and that an intermediate enzyme-substrate complex consisting of one molecule each of the enzyme and the substrate is probably formed before the substrate is hydrolysed.

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52-43 Printed at The Bangalore Press, Mysore Road, Bangalore City, by G. Srinivasa Rao, Superintendent and Published by The Indian Academy of Sciences, Bangalore