THE NATURE OF PROTEINASES OF THERMOPHILIC BACTERIA

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Received April 29, 1946

The proteolytic enzymes of bacteria and molds have not been sufficiently studied and the nature and type of microbial proteinases are still imperfectly understood. An exact knowledge of the type of bacterial proteinases should be of interest not only from the theoretical point of view but also in its application to the study of attack and degradation of animal and plant tissues, the study of proteolytic phenomenon in soil, the investigations on storage and deterioration of foodstuffs and the investigations on some industrial processes. While most workers are agreed that bacterial proteinases cannot be classified with the pepsinases, opinion is divided as to whether these proteinases are of papainase type or tryptase type.

Dernby and Blanc (1921) had found that culture filtrates of several anærobes bacteria digest gelatin optimally at pH 6 from which they concluded that the proteinase is of a tryptic nature. Kendall and Keith (1926) and Schierge (1926) working with *Bacillus proteus* and *B. coli* respectively have also concluded that their proteinases are of tryptic nature, the optimum hydrogen-ion concentration in the latter case was stated to be at pH 6·0 to 6·6. These conclusions must be revised because tryptases optimally hydrolyse markedly cationic form of protein. Walbum and Reymann (1934) and Bessey and King (1934) have obtained conflicting results with *Clostridium histolyticum*. In several papers Maschmann (1937, 1938) has published his results with *Bacillus pyocyaneus, B. prodigiosus, B. fluorescence, B. perfrigens, B. histolyticum* and *B. botulinus*. Many of these micro-organisms produce a proteinase whose optimum pH is 7 and is activated in some cases by hydrocyanic acid and by thiol compounds. Weil and Kocholaty (1937) and Kocholaty, Weil and Smith (1938) have studied *Clostridium histolyticum* and by measuring proteolytic activity by estimating the liberation of free α-amino acids they have found that *Cl. histolyticum* produces a proteinase which is active optimally at pH 7, is activated by thiol compounds and is inert towards enterokinase.

The micro-organisms studied by the present author were the typical thermophilic bacteria *Bacillus thermophilus, B. ærothermophilus* and *B. thermoacidurans.* Cultures of these were obtained from the Lister Institute, London. The thermophilic bacteria can grow at high temperatures, often close to the coagulation temperature of their albumins. This renders them an intriguing subject for study. These bacteria are widely distributed in soil, etc., and their proteolytic activities are called into play in several processes of importance in soil science, in agriculture and in industry. Clark and Tanner (1937) and McMaster (1934–5) have shown the importance of thermophiles in food preservation. A commonly occurring spoilage of soya beans has been ascribed to the proteolytic action of *B. thermophilus* by Rokusho and Fukutome (1937). Thermophilic bacteria are active agents in manure fermentation, see for example, Dunez (1933) and Damon and Feirer (1925). Proteolytic thermogenesis of wool has been studied by Barker (1929) and according to James (1928) nitrogen metabolism and thermogenesis are interrelated. The harmful heating up of hay, fodder, textile materials and thermophilic fermentation in the processing of tobacco, cocoa and coffee are well known and thermophilic bacteria undoubtedly play a part in these.

All earlier investigations on the nature of microbial proteinases are based on the determination of pH optimas and response towards papainase and tryptase activators and inhibitors. In the present investigations, in addition to studying these aspects, an attempt has been made, by duplicate enzyme experiments, to determine whether or not the peptide bonds, in the protein molecule, hydrolysed by the bacterial proteinases are identical to those hydrolysed by either pepsin, papain or trypsin or *vice versa.*

The experimental technique adopted was quite simple. The bacteria were grown in nutrient broth by incubation at 50° C. for forty-eight hours. The cultures were centrifuged and filtered through Chamberland candles. This yielded cell-free proteolytically powerful filtrates free from peptonase or polypeptidase. Substrates used were gelatin, egg albumin and casein made into aqueous solution at the appropriate pH with McIlvaine's citrate-phosphate buffer. Proteolytic hydrolysis was allowed to proceed at 40° C. in presence of a drop of toluene. 2 ml. of the proteinase filtrate were used per 20 ml. of substrate solution. In case of gelatin the initial stages of proteolysis were followed viscometrically. With egg albumin the initial stage of proteolysis was followed by precipitating the unaltered protein by boiling at the isoelectric point or by precipitating the unaltered protein, meta-proteins and albumoses in 4% trichloracetic acid followed by estimation of the fraction which was soluble under these conditions.

In all cases the increase in free α-amino groups, during incubation at 40° C. for forty-eight hours, was estimated by the micro Van Slyke method or by the titration method.

pH Optimum of Proteinases of Thermophilic Bacteria

2 ml. of proteinase solution were added to 20 ml. of 3% gelatin solution at pH ranging from 3 to 10. Initial rate of hydrolysis was calculated by extrapolating viscosity time curves to zero time and also per cent. fall in initial viscosity in 30 minutes was determined. Finally the increase in α-amino acids during incubation at 40° C. for 48 hours was estimated by Van S yke's method. In case of egg albumin 2 ml. of the proteinase solution were added to 20 ml. of the protein solution containing 1·3 to 1·4 mgm. of organic nitrogen per ml. The nitrogenous matter not precipitated by boiling at isoelectric point or in 4% trichloracetic and the amount of free α-amino nitrogen were estimated before and after incubation. In case of casein also proteolysis was followed by estimation of free α-amino acids. In all cases pH curves were plotted from which the values for optimum pH were derived. These were as follows:—

Table I

Proteinase of	Gelatin	Egg albumin	Casein
B. thermophilus ..	8·3	8·0	7·7
B. aerothermophilus ..	7·7	8·0	7·3
B. thermoacidurans ..	8·1	8·0	7·5

Thus all these proteinases are optimally active in the alkaline region, *i.e.*, they hydrolyse the cationic form of proteins. In this respect therefore they resemble the tryptases rather than the papainases or the pepsinases.

Effect of Papainase Activators on the Proteinases of Thermophilic Bacteria

The substrate used was 3% gelatin solution prepared at the respective pH in McIlvaine's citrate-phosphate buffer. The enzyme solution was incubated with the activating reagent at 30° C. half an hour before mixing with the substrate. In case of hydrogen cyanide or hydrogen sulphide the gas was bubbled through the cold enzyme solution for a few minutes and the solution was then incubated in a stoppered test-tube at 30° for half an hour.

TABLE II

Reagent	Concentration	% Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen mgm./10
		B. thermophilus		
None ..		6·72	48·05	5·85
Hydrogen sulphide ..		6·01	42·10	5·27
Hydrogen cyanide ..		6·05	42·60	5·29
Cystein ..	M/250	6·63	46·90	5·29
		B. aerothermophilus		
None ..		6·03	41·05	5·27
Hydrogen sulphide ..		5·90	40·00	5·21
Hydrogen cyanide ..		5·96	40·65	5·30
Cystein ..	M/250	6·00	41·00	5·29
		B. thermoacidurans		
None ..		6·35	45·90	5·91
Hydrogen sulphide ..		6·00	42·55	5·32
Hydrogen cyanide ..		6·27	44·95	5·45
Cystein ..	M/250	5·32	45·35	5·57

It is therefore obvious that the typical papain activators do not activate the proteinases of thermophilic bacteria. In fact there is a very slight inhibition in some cases.

EFFECT OF PAPAINASE INHIBITORS ON THE PROTEINASES OF THERMOPHILIC BACTERIA

The experimental and analytical methods used were the same as in the previous experiment.

TABLE III

Reagent	Concentration	% Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen mgm./10 ml.
		B. thermophilus		
None ..		6·25	46·70	5·83
Iodacetic acid ..	M/250	6·18	47·90	5·78
Hydrazine ..	M/200	6·35	46·05	5·79
Copper sulphate ..	M/200	6·29	47·00	5·85
		B. aerothermophilus		
None ..		6·20	41·55	5·28
Iodacetic acid ..	M/250	6·15	41·25	5·35
Hydrazine ..	M/200	6·22	41·30	5·22
Copper sulphate ..	M/200	6·18	41·90	5·29
		B. thermoacidurans		
None ..		6·35	46·15	5·59
Iodacetic acid ..	M/250	6·36	46·10	5·62
Hydrazine ..	M/200	6·32	46·50	5·57
Copper sulphate ..	M/200	6·30	46·20	5·60

Obviously therefore the typical papainase inhibitors have no significant effect on the proteinases of thermophilic bacteria.

Effect of Enterokinase on the Proteinases of Thermophilic Bacteria

Enterokinase was prepared freshly from swine duodenum and was freed from trypsin by fractional precipitation with ammonium sulphate. Its activity was checked against crude pancreatic extracts. The preparation showed negligible proteolytic activity by itself when tested against gelatin. For examining its effect on microbial proteinases the powder was mixed with the proteinase solution and the mixture was incubated at 30° C. for one hour with occasional shaking.

Table IV

Proteinase of	Without enterokinase			With enterokinase		
	% Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen mgm./10 ml.	% Fall in initial viscosity in first 5 minutes	% Fall in initial viscosity in 30 minutes	Increase in α-amino nitrogen mgm./10 ml.
B. thermophilus	6·83	47·50	6·11	6·84	47·05	6·07
B. aerothermophilus	6·44	45·95	5·65	6·40	46·15	5·59
B. thermoacidurans	6·05	43·15	5·31	6·00	43·00	5·38

The above data shows that enterokinase, the specific activator of the tryptases, has no effect on the proteinases of thermophilic bacteria.

Effect of Pepsin, Papain and Trypsin on Gelatin Solutions Previously Hydrolysed by the Proteinases of Thermophilic Bacteria

Most proteinases do not open up all the peptide bonds in the protein molecule. For example papain can open up more peptide bonds after a gelatin solution has been digested to completion with an excess of trypsin or pepsin and *vice-versa*. The same is the case when trypsin is allowed to act on a gelatin solution previously hydrolysed to completion by an excess of pepsin and *vice-versa*. In the present experiment 200 ml. each of 3% gelatin solution were incubated with 40 ml. each of highly powerful solutions of the proteinases of ***Bacillus thermophilus***, ***B. aerothermophilus*** and ***B. thermoacidurans*** respectively for several days at optimum pH till there was no

further increase in free α-amino acids. Each solution was then divided into four portions and the pH of three of these four portions was re-adjusted to the respective optimum pH of pepsin, papain and trypsin. With the fourth portions the pH was restored to the original starting pH as there had been a slight fall in pH during the prolonged proteolysis. The four lots of 50 ml. each comprising each of the three sets were then subjected to the action of pepsin, papain, trypsin and fresh culture filtrate of the same bacteria respectively. Incubation was carried out at 40° C. for 36 hours in presence of toluene.

TABLE V

Increase in free α-amino nitrogen, mgm./10 ml.

First proteinase	Second proteinase				
	Blank (distilled water)	Same as first	Pepsin	Papain	Trypsin
B. thermophilus ..	Nil	0·13	1·38	3·95	2·05
B. aerothermophilus ..	,,	0·11	1·02	4·01	2·86
B. thermoacidurans ..	,,	0·08	1·40	3·13	2·95
Pepsin ..	,,	0·06	0·06	4·02	3·39
Papain ..	,,	0·09	1·52	0·09	3·22
Trypsin ..	,,	0·10	1·30	3·09	0·10

It is therefore obvious that gelatin contains some peptide bonds which cannot be opened by the microbial proteinases but which are available for attack by pepsin, papain and by trypsin. From this it would appear that the type specificity and mode of attack of the proteinases of the thermophilic bacteria is different from that of either pepsin, papain or trypsin, just as the type specificity and mode of attack of pepsin, papain and trypsin is different from that of each other.

It was now decided to investigate if either one or more of the three typical proteinases pepsin, papain and trypsin can hydrolyse all those peptide groups which are hydrolysable by the proteinases of the thermophilic bacteria.

EFFECT OF PROTEINASES OF THERMOPHILIC BACTERIA ON PEPTIC, PAPAIN AND TRYPTIC DIGESTS OF GELATIN

The experimental methods were the same as in the previous experiment except that the positions of bacterial proteinases on the one hand and those of pepsin, papain and trypsin on the other were reversed. In order to remove any possibility of doubt the first series of hydrolysis was conducted

with papain activated with hydrogen cyanide and with trypsin activated with enterokinase.

TABLE VI

Increase in α-amino nitrogen, mgm./10 ml.

First proteinase	Second proteinase				
	Blank (distilled water)	Same as first	*B. thermophilus*	*B. aerothermophilus*	*B. thermoacidurans*
Pepsin ..	Nil	0·12	5·12	4·44	4·72
Papain–HCN ..	,,	0·04	3·69	2·95	2·35
Trypsin enterokinase ..	,,	0·06	3·45	3·67	2·95

Therefore the bacterial proteinases can hydrolyse certain peptide groups that can be hydrolysed neither by pepsin nor by papain or trypsin. From Table VI it appears that the increase in α-amino nitrogen in the second hydrolysis is greater with all three bacterial proteinases if the first hydrolysis is carried out with pepsin. This accords with the fact that pepsin is mainly a disaggregating enzyme.

DISCUSSION AND SUMMARY

The nature and type of bacterial proteinases has been the subject of a certain amount of discussion in the past and conflicting opinions have been expressed as to whether these proteinases are peptic, papainase or tryptic in nature. Previous investigators have studied the degree and optimum pH of hydrolysis and activation-inhibition behaviour of the bacterial proteinases and have attempted to classify them with pepsinases, papainases or tryptases. The results of studies of this type afford an insight into the nature and properties of the proteinases. In the present investigations a study has been made of the optimum pH and activation-inhibition properties of the bacterial proteinases and in addition attempts have been made to investigate if the bacterial proteinases attack peptide bonds which are identical with or entirely or partially different from those hydrolysed by pepsin, papain and trypsin or *vice-versa*. All the three bacterial proteinases studied here hydrolyse gelatin, casein and egg albumin optimally in the alkaline region, *i.e.*, like the tryptases they hydrolyse the cationic form of proteins. They are not activated by papain activators and are not inhibited by papain inhibitors. Likewise they do not respond to trypsin activators. Gelatin solutions which have already been subjected to prolonged hydrolysis by pepsin, papain or trypsin and which cannot be further hydrolysed by these three proteinases are further

hydrolysed by the bacterial proteinases. Conversely gelatin solutions which have already been hydrolysed to completion by the bacterial proteinases can be further hydrolysed by pepsin, papain or trypsin. This shows that the peptide bonds opened up by the bacterial proteinases are at least partially different from those hydrolysed by either pepsin, papain or by trypsin. From these results it is obvious that out of the three main categories of proteinases, *i.e.*, the pepsinases, papainases and tryptases the evidence in favour of the bacterial proteinases studied here belonging to the tryptic class of proteinases is relatively the strongest. But there are also points of difference between the bacterial proteinases and tryptases. The former do not respond to trypsin activators. This may be due either to the fact that the bacterial proteinases are not of tryptic nature or else that when they are obtained in the culture filtrates they are already in the fully active state. The results of duplicate enzyme experiments have shown that the bacterial proteinases hydrolyse peptide bonds which are at least partially different from those hydrolysed by trypsin and *vice-versa*. On the whole it would appear to be more satisfactory to avoid classifying microbial proteinases with any of the three main groups of proteinases but rather to leave them in a class by themselves.

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