CARBOHYDRATES AND MICROBIAL PROTEINASES

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The question whether carbohydrates affect proteinase formation by micro-organisms is of interest in soil and in agricultural biochemistry. It is generally believed that presence of carbohydrates suppresses proteolytic activity and proteinase formation by micro-organisms. This inhibitive effect is usually explained by assuming that carbohydrates are preferentially utilised by the growing cells as their source of energy and carbon and that in their presence the micro-organisms attack proteins or peptone solely for obtaining the nitrogen with which to build their protoplasm proteins. The enzymic system of the cells being adaptive in nature the amount of protein decomposing enzyme formed is therefore considerably reduced. See, for example, Kendall (1922). Console and Rahn (1938) have shown that filtrates from cultures of *Bacillus subtilis* were proteolytically inactive when glucose was present in the growth medium. According to Wilson (1930), however, in case of *B. pyocyaneus*, proteinase formation was not affected by the presence of carbohydrates in the growth medium.

The experimental technique adopted by most previous investigators to study the effect of carbohydrates on proteinase formation seems to have been to grow the micro-organism in media containing the carbohydrates and to test the culture filtrates for proteolytic activity. The present investigations were carried out with the soil aerobe *Bacillus cereus* and with the typical thermophilic bacteria *B. thermophilus*, *B. aerothermophilus* and *B. thermoacidurans* and the conclusions are here published in view of their somewhat divergent nature from those of earlier investigators. The bacteria selected for the present study are all fairly widespread in soil, etc., and are responsible for a large number of enzymic transformations which are of importance in soil and agricultural biochemistry and in most thermophilic fermentations.

The organisms were grown in nutrient broth containing the respective carbohydrate in a 2% final concentration. The cultures were incubated for forty-eight hours at 50° C. for the thermophilic bacteria and at 37° C. for *Bacillus cereus* after which they were centrifuged and filtered through chamberland candles yielding cell-free filtrates whose proteolytic activity was tested on gelatin solutions by following the rapid decrease

in their viscosity and by measuring the increase in free α-amino acids by the micro Van Slyke method. In the viscometric method the initial velocity of hydrolysis was calculated by extrapolating viscosity/time curves to zero time and also the per cent. fall in initial viscosity in thirty minutes was calculated from the same set of observations.

TABLE I

Effect of Carbohydrates

Carbohydrate in 2% final concentration	Initial velocity of hydrolysis, % fall in initial viscosity in first 5 minutes	% Fall in initial viscocity in 30 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
	Bacillus cereus		
Nil ..	7·76	54·65	7·01
Dextrose ..	0·81	4·95	0·66
Lævulose ..	0·32	2·60	0·00
Sucrose ..	4·11	29·45	3·87
Glycerol ..	7·05	48·60	6·73
Dextrin ..	7·41	50·30	6·83
Starch ..	7·83	53·90	7·09
	Bacillus thermophilus		
Nil ..	6·79	49·85	6·13
Dextrose ..	0·41	4·15	0·73
Lævulose ..	0·17	1·75	0·89
Sucrose ..	3·23	26·10	2·93
Glycerol ..	5·87	40·05	5·07
Dextrin ..	6·48	47·95	5·79
Starch ..	6·77	50·15	6·03
	Bacillus aerothermophillus		
Nil ..	7·35	52·45	6·49
Dextrose ..	0·63	5·20	0·72
Lævulose ..	0·19	6·05	0·00
Sucrose ..	2·97	20·10	2·13
Glycerol ..	5·83	39·75	5·07
Dextrin ..	6·12	43·15	5·31
Starch ..	6·89	48·60	6·12
	Bacillus thermoacidurans		
Nil ..	7·07	50·10	6·31
Dextrose ..	0·35	3·20	0·71
Lævulose ..	0·32	3·05	0·79
Sucrose ..	2·31	18·10	1·82
Glycerol ..	5·82	38·75	5·13
Dextrin ..	6·47	47·30	5·68
Starch ..	6·88	49·95	6·20

The above results show that the presence of carbohydrates in the growth medium results in a decrease of the proteinase activity of cell-free filtrates. The effectiveness of the carbohydrates in depressing proteinase activity of the filtrates is in the following increasing order: starch, dextrin, glycerol, sucrose, dextrose and lævulose and this is also the increasing order of availability of these carbohydrates for attack and assimilation by the bacteria. The bacterial growth in dextrose, lævulose and in sucrose broths was much

more profuse than in plain broth, yet cell-free filtrates from the cultures in the former media showed negligible proteolytic activity.

TABLE II

Effect of dextrose at different concentrations

Carbohydrate		Mgm. dry weight of cells per 100 ml. of culture	Initial velocity, % fall in initial viscosity in first 5 mintues	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
	%	*Bacillus cereus*		
Nil		50·5	7·32	6·41
Dextrose	0·10	68·0	7·48	6·57
,,	0·25	115·7	7·36	6·89
,,	0·50	149·0	7·95	7·01
,,	0·75	173·5	6·30	5·45
,,	1·00	188·3	3·15	2·72
,,	1·50	196·5	1·52	1·17
,,	2·00	200·4	0·63	0·89
		Bacillus thermophilus		
Nil		15·3	6·37	5·41
Dextrose	0·10	30·0	6·52	5·57
,,	0·25	46·7	7·01	5·89
,,	0·50	69·3	7·00	5·95
,,	0·75	80·2	5·43	4·61
,,	1·00	92·4	2·15	1·58
,,	1·50	100·0	1·03	0·95
,,	2·00	106·5	0·54	0·60
		Bacillus aerothermophilus		
Nil		43·0	6·15	5·27
Dextrose	0·10	68·5	6·38	5·36
,,	0·25	89·6	6·75	5·83
,,	0·50	117·5	6·89	5·96
,,	0·75	142·3	6·01	5·02
,,	1·00	166·2	3·82	3·17
,,	1·50	183·4	2·41	1·78
,,	2·00	190·0	0·95	0·88
		Bacillus thermoacidurans		
Nil		48·1	6·37	5·49
Dextrose	0·10	60·4	6·55	5·56
,,	0·25	90·2	6·82	5·88
,,	0·50	120·0	6·70	6·02
,,	0·75	139·5	6·01	5·00
,,	1·00	150·8	3·15	2·32
,,	1·50	170·5	1·68	0·95
,,	2·00	190·9	0·85	0·43

As is evident from the above tables dextrose and lævulose upto about 0·5% concentration actually increase the proteinase content in the culture filtrates, probably because of increased cell formation. Beyond this concentration the effect of increased cell formation is masked by the inhibitive effect of the sugars on proteinase activity of the filtrates, till when the concentration of sugars reaches 2% the filtrates have negligible proteolytic activity. The amount of bacterial mass produced in each case was measured

TABLE III

Effect of lævulose at different concentrations

Carbohydrate		Mgm. dry weight of cells per 100 ml. of culture	Initial velocity, % fall in initial viscocity in first 5 minutes	Increase in α–amino nitrogen, mgm./10 ml. in 48 hours
	%	*Bacillus cereus*		
Nil	..	50·5	7·32	6·41
Lævulose	0·10 ..	85·0	7·68	6·80
,,	0·25 ..	130·2	7·89	7·02
,,	0·50 ..	165·5	7·80	6·89
,,	0·75 ..	180·4	6·16	5·83
,,	1·00 ..	193·0	3·20	2·24
,,	1·50 ..	205·6	1·23	1·85
,,	2·00 ..	210·4	0·32	0·00
		Bacillus thermophilus		
Nil	..	15·3	6·37	5·41
Lævulose	0·10 ..	35·6	6·49	5·53
,,	0·25 ..	65·2	6·75	6·02
,,	0·50 ..	80·3	6·60	6·01
,,	0·75 ..	90·5	3·85	2·95
,,	1·00 ..	100·4	2·24	1·68
,,	1·50 ..	115·4	1·11	0·95
,,	2·00 ..	120·0	0·38	0·05
		Bacillus aerothermophilus		
Nil	..	48·1	6·37	5·49
Lævulose	0·10 ..	100·3	6·63	5·72
,,	0·25 ..	130·2	6·95	6·15
,,	0·50 ..	135·8	7·13	6·20
,,	0·75 ..	150·5	5·33	5·00
,,	1·00 ..	170·5	4·38	3·85
,,	1·50 ..	183·2	2·35	1·82
,,	2·00 ..	198·6	0·36	0·08
		Bacillus thermoacidurans		
Nil	..	43·0	6·15	5·27
Lævulose	0·10 ..	..	6·48	..
,,	0·25 ..	..	6·92	6·10
,,	0·50 ..	120·4	6·95	6·05
,,	0·75 ..	150·6	5·63	..
,,	1·00 ..	..	..	..
,,	1·50 ..	..	..	..
,,	2·00 ..	206·4	0·27	0·13

by filtering cultures through the filtration tube of a previously weighed micro-beaker and re-weighing after washing the bacterial mass and drying over phosphorus pentoxide in vacuum at room temperature.

The above findings confirm the opinions of the earlier workers, namely that carbohydrates restrict proteinase formation. But the question now arises whether the effect of carbohydrates is actually that of prevention of proteinase formation, or whether they simply prevent the diffusion of the proteinase from the dead or living cells into the surrounding medium or whether presence of carbohydrates in the growth medium renders the proteinase formed incapable of functioning in the absence of the living cells.

Experiments were therefore conducted to determine the proteinase content of whole cultures, cells alone and cell-free filtrates, when say lævulose was present in the medium at start. For determining the proteinase content of cells the latter were reaped by centrifuging and were re-suspended in water to give the same volume as that of the culture they were obtained from. In every case 2 ml. of the whole culture, cell suspension or cell-free filtrate were mixed with 10 ml. of the gelatin substrate and viscometric observations and Van Slyke α-amino nitrogen estimations were carried out as usual.

TABLE IV

Cultures in media containing 2% lævulose

Organism	Source of proteinase	Initial velocity, % fall in initial viscosity in first 5 minutes	Increase in α-amino nitrogen, mgm./10 m . in 48 hours
Bacillus cereus ..	Whole culture	6·38	5·49
	Cell-free filtrate	0·41	0·08
	Cells alone	6·01	5·32
Bacillus thermophilus ..	Whole culture	5·68	4·87
	Cell-free filtrate	0·22	0·00
	Cells alone	5·11	4·32
Bacillus aerothermophilus ..	Whole culture	6·27	5·43
	Cell-free filtrate	0·11	0·03
	Cells alone	6·01	5·29
Bacillus thermoacidurans ..	Whole culture	6·13	5·33
	Cell-free filtrate	0·29	0·11
	Cells alone	5·75	5·13

TABLE V

Cultures in media containing 2% dextrose

Organism	Source of proteinase	Initial velocity, % fall in initial viscosity in first 5 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
Bacillus cereus ..	Whole culture	5·82	5·32
	Cell-free filtrate	0·02	0·00
	Cells alone	5·75	5·22
Bacillus thermophilus ..	Whole culture	5·93	5·21
	Cell-free filtrate	0·18	0·15
	Cells alone	5·80	5·00
Bacillus aerothermophilus ..	Whole culture	6·50	6·12
	Cell-free filtrate	0·32	0·27
	Cells alone	6·29	5·39
Bacillus thermoacidurans ..	Whole culture	6·01	5·35
	Cell-free filtrate	0·20	0·05
	Cells alone	5·65	5·02

It is obvious therefore that dextrose and lævulose do not actually prevent proteinase formation but that the proteinase produced either does

not pass out into the cell-free medium or that it does not function in the absence of living cells.

Attempts were then made to see if any process could be devised to make the proteinase diffuse out of the cells. Autolysing the cells in various ways failed to achieve the purpose. Autolysis merely resulted in destruction of the proteinase within the cells as is obvious from Table VI.

TABLE VI

Treatment	*Bacillus cereus*, % fall in initial viscosity in first 5 minutes	*Bacillus cereus*, increase in α-amino N. mgm./10 ml. in 48 hours	*B. thermophilus*, % fall in initial viscosity in first 5 minutes	*B. thermophilus*, increase in α-amino N. mgm./10 ml. in 48 hours	*B. aerothermophilus*, % fall in initial viscosity in first 5 minutes	*B. aerothermophilus*, increase in α-amino N. mgm./10 ml. in 48 hours	*B. thermoacidurans*, % fall in initial viscosity in first 5 minutes	*B. thermoacidurans*, increase in α-amino N. mgm./10 ml. in 48 hours
1. Suspensions of cells, untreated	7·32	6·48	6·69	5·86	7·11	5·93	6·95	6·10
2. Autolysed with toluene for 8 hours at 40° C., autolysate	0·05	0·00	0·00	0·00	0·00	0·00	0·00	0·00
3. Same as 2, cells ..	1·31	0·93	0·86	0·52	0·43	0·00	1·83	1·01
4. Autolysed with chloroform for 4 hours at 40° C., autolysate	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
5. Same as 4, cells ..	2·41	1·43	1·42	0·83	1·86	1·01	0·93	0·05
6. Glycerol-phosphate, 3 hours at room temp., autolysate	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
7. Same as 6, cells ..	3·93	3·04	4·15	3·29	3·00	2·17	3·41	2·13
8. Autolysed with toluene, 4 hours at 20° C., autolysate	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
9. Same as 8, cells ..	1·60	1·32	2·31	1·05	1·43	0·62	0·71	0·00
10. Phenole — gelatine, 6 hours at 37° C., autolysate	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
11. Same as 10, cells ..	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

It therefore seems that killing of cultures and autolysis results in destruction of the proteolytic activity of these cultures. For the sake of comparison these experiments were repeated with cultures grown in plain nutrient broth, *i.e.*, in the absence of any carbohydrate and the results summarised in Table VII show that destruction of the cell life process in carbohydrate-free cultures does not result in destruction of the proteolytic activity. Autolysis of these carbohydrate-free cultures was always carried out at 40° C. under toluene for eight hours.

TABLE VII

Organism	Culture	Initial velocity of hydrolysis, % fall in initial viscosity in first 5 minutes	Increase in α-amino nitrogen, mgm./10 ml. in 48 hours
Bacillus cereus ..	Alive	7·32	6·58
	Autolysed	7·18	6·53
B. thermophilus ..	Alive	6·88	6·07
	Autolysed	6·81	6·00
B. aerothermophilus ..	Alive	6·92	6·17
	Autolysed	7·01	6·13
B. thermoacidurans ..	Alive	6·13	5·32
	Autolysed	6·10	5·37

DISCUSSION AND SUMMARY

With the four bacteria *Bacillus cereus, B. thermophilus, B. aerothermophilus* and *B. thermoacidurans* here examined, presence of carbo-hydrates in th growth media results in a decrease in the proteolytic activity of the culture filtrates. The depressing effect of the carbohydrates is proportional to the degree of availability of the carbohydrate for attack by the bacteria. Although the proteolytic activity of the culture filtrates is descreased proteinase formation is not prevented or depressed by the presence of carbohydrates in the growth medium. The cells reaped from cultures prepared in carbohydrate media are very rich in proteinase but it is very difficult to extract the proteinase from the cells. The effect of carbohydrates is thus to make the proteinase intimately combined with some cell constituents and is not to prevent or hinder the formation of proteinase.

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