THE EXOCELLULAR BACTERIOLYTIC SYSTEM OF SOIL ACTINOMYCES

III. THE SEPARATION AND CHARACTERISATION OF THE PROTEOLYTIC ENZYMES

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In Parts 1 and 2 of this series (JONES, SWALLOW AND WEBB[1]; MUGGLETON AND WEBB[2]) it was shown that the lysis of either killed Gram-negative cells, or of the Gram-negative forms of Gram-positive organisms by an *Actinomyces Sp.* culture filtrate, is brought about by a proteolytic enzyme system consisting of a proteinase and a peptidase. Of these enzymes, the proteinase hydrolyses high molecular weight proteins and is responsible for the initial breakdown of the proteins and lysis of the dead Gram-negative cell.

The present communication describes an investigation of methods for the separation of the proteolytic enzymes of the bacteriolytic system of the *Actinomyces Sp.* In addition, the enzymes have been characterised to some extent by a study of their action on synthetic substrates.

EXPERIMENTAL

Preparation of the enzymes

Culture filtrates from the *Actinomyces* strain "A" were purified by dialysis, concentrated by pervaporation and dried from the frozen state as described in Part 2.

The activity of the proteinase was measured viscosimetrically against a casein substrate and that of the peptidase by the formol titration method with peptone as substrate. Enzymic activities under these conditions were expressed in the arbitrary units previously defined (MUGGLETON AND WEBB[2]). With synthetic peptides as substrates, the activities of the enzymes were expressed in terms of the percentage hydrolysis of the given substrate in a given time.

Separation of the enzymes

1. *Preferential deactivation of the peptidase by heat*

When a solution of the enzymes was maintained at 60°, the peptidase was denatured to a greater extent than the proteinase (Table I). Inactivation of the peptidase under these conditions was observed either with solutions of the purified enzymes or with the crude culture filtrate, and was complete in 45–60 minutes. At the end of this period the residual proteinase activities in the crude filtrate and purified solution amounted to about 50% and 20% of the initial activities respectively.

References p. 536.

TABLE I

PREFERENTIAL INACTIVATION OF THE PEPTIDASE OF THE ACTINOMYCES BACTERIOLYTIC SYSTEM AT 60°

At the time intervals shown in the table, samples of the enzyme solution were removed, cooled to room temperature, and assayed for proteinase and peptidase activities.

Time of heating (min)	*Crude culture filtrate*		*Purified enzyme solution*	
	Proteinase U/ml	*Peptidase U/ml*	*Proteinase U/ml*	*Peptidase U/ml*
0	122	82	118	79
15	83	41	45	61
30	76	11	39	20
45	65	0	25	5
60	50	0	20	0
90	44	0	18	0
120	40	0	20	0

Both the crude culture filtrate and the purified enzyme solution after 60 min at 60° (*i.e.*, free from peptidase activity) exhibited a marked lytic activity against a suspension of heat-killed *Bact. lactis aerogenes*.

2. Separation of the enzymes by fractional precipitation

The concentrated, freeze-dried Actinomyces enzyme preparation (120 mg) in water (120 ml) was fractionated with acetone as described by JONES, STACEY AND WEBB[3]. Precipitates were collected at acetone concentrations of 40, 50, 60 and 80%. Each precipitate was dissolved in water (5 ml). The residual 80% acetone solution was concentrated *in vacuo* to 20 ml to remove the acetone. Aliquots of these solutions were analysed for total nitrogen by the micro-Kjeldahl method. The proteinase and peptidase activities of the fractions were determined in the usual way.

The results (Table II) show that complete precipitation of either enzyme did not occur at any of the above acetone concentrations. The precipitate which separated at an acetone concentration of 60% contained the greatest amount of both enzymes and, as shown by the figures for enzyme activity/mg N, resulted in considerable purification.

TABLE II

FRACTIONATION OF PURIFIED ENZYME SOLUTION BY ACETONE PRECIPITATION

Fraction	*Total enzyme units in fraction*		*Units/mg Total nitrogen*		*Ratio* $\frac{Proteinase}{Peptidase}$
	Proteinase	*Peptidase*	*Proteinase*	*Peptidase*	
Initial solution	4,120	1,720	5,920	2,470	2.68
40% acetone ppt.	432	110	5,470	1,392	3.93
50% acetone ppt.	599	410	4,750	3,259	1.45
60% acetone ppt.	870	520	13,800	8,250	1.67
80% acetone ppt.	1,912	320	5,500	919	5.97

Total units recovered: Proteinase 4,493 U; Peptidase 1,790 U

References p. 536.

3. *Fractionation of the enzymes by precipitation with ammonium sulphate and adsorption on foam*

Attempts to separate the two enzymes by fractional precipitation with ammonium sulphate were unsuccessful, since both enzymes, presumably of low molecular weight, required high concentrations of the salt for complete precipitation. Furthermore, repeated fractional precipitation with ammonium sulphate was accompanied by a considerable loss of enzyme activity.

It was shown in a previous communication (JONES, SWALLOW AND WEBB[1]) that partial separation of the Actinomyces enzymes could be obtained by adsorption on foam according to the method of SCHUTZ[4]. Whereas this technique was applicable directly to broth filtrates then used, the synthetic medium filtrates and solutions of the purified enzymes used in the present investigation could not be induced to foam. In order to produce a stable foam it was necessary to decrease the solubility of the enzymes in the bulk of the solution by the addition of ammonium sulphate as follows:

Ammonium sulphate (20 g) was added to a solution of 100 mg of purified, freeze dried Actinomyces enzymes in distilled water (48 ml) (ca 0.5 saturation). The solution was adjusted to pH 6.7 with sodium hydroxide and introduced into the foam apparatus. A slow current of nitrogen was passed through the solution such that five fractions (f1–f5), each of about 5 ml volume, were collected at intervals of approx. 5 minutes. These fractions were dialysed against running tap water until free from sulphate (36 hours) and concentrated to 6 ml *in vacuo* at 35°. (Final pH 6.52–6.58). In addition, the solution (f6; 33 ml) remaining in the apparatus after foaming was dialysed and concentrated to 12 ml. The proteinase and peptidase activities of these fractions were determined as usual. The results (Table III) indicate that the proteinase is concentrated on the foam to a greater extent than the peptidase.

TABLE III

PARTIAL SEPARATION OF THE PROTEOLYTIC ENZYMES IN 0.5 SATURATED AMMONIUM SULPHATE ON FOAM

Fraction	*Enzyme activity (U/ml)*		*Ratio* $\frac{Proteinase}{Peptidase}$
	Proteinase	*Peptidase*	
Initial solution	151	106	1.43
f1	213	127	1.68
f2	178	125	1.43
f3	119	101	1.18
f4	67	94	0.72
f5	49	65	0.76
f6 (Residual soln)	2	17	0.15

After the estimations summarised in Table III, fractions f1–f6 were combined. The resulting solution (24 ml) was adjusted to ca 0.33 saturation of ammonium sulphate by the addition of 6 g of the salt, and "foamed" as before. Two fractions (f1a and f2a each of about 4 ml volume were collected before the solution ceased to foam. A further 2 g of ammonium sulphate were added and two additional fractions (f3a and f4a), each of about 4 ml volume, collected. These fractions, together with the residual solution (f5a) were dialysed, concentrated and assayed for proteinase and peptidase activities.

References p. 536.

The results (Table IV) show that the proportions of the two enzymes which foam over are dependent upon the ammonium sulphate concentration. At 0.33 saturation of the salt and at 0.5 saturation (based on the amount of ammonium sulphate added), the proteinase has the greater surface activity and is concentrated in the first fractions (f1a and f3a), whereas the peptidase is concentrated in the residual solution (f5a). Fraction f3a was free from the peptidase and furnished a solution which had proteinase activity alone.

TABLE IV

THE SEPARATION OF THE PROTEINASE ENZYME ON FOAM

Fraction	*Enzyme activity (U/ml)*		*Ratio* $\frac{Proteinase}{Peptidase}$
	Proteinase	*Peptidase*	
Initial solution	91	82	1.12
f1a	106	50	2.12
f2a	28	32	0.88
f3a	68	0	—
f4a	107	8	13.4
f5a	14	40	0.35

TABLE V

SEPARATION OF THE PROTEOLYTIC ENZYMES BY FOAMING AFTER PRELIMINARY FRACTIONATION WITH ACETONE

Fraction	*Total units of activity*		*Ratio* $\frac{Proteinase}{Peptidase}$
	Proteinase	*Peptidase*	
f1b	65	0	—
f2b	24	60	0.4
f3b	135	60	2.25
f4b	132	44	2.99
f5b	40	90	0.45
f6b (Residual soln)	0	60	—

4. *Fractionation of the enzymes by acetone precipitation and adsorption on foam*

From the results of Table II, it is apparent that the solution remaining after fractional precipitation with acetone contains a greatly increased proportion of proteinase. In view of the above results, this solution was adjusted to 0.5% saturation with ammonium sulphate and subjected to foaming. Five foam fractions (f1b–f5b) each of c. 4 ml in volume were collected at 5 minute intervals. These, together with the residual solution (f6b), were dialysed to remove ammonium sulphate and examined for proteinase and peptidase activities. Under these conditions complete separation of the enzymes was obtained (Table V). The initial foam fraction (f1b) contained the proteinase alone, whereas the residual solution (f6b) contained only the peptidase.

5. *Separation of the enzymes by dialysis*

It was noted in Part 2 of this series that a gradual loss of enzyme activity occurred during the dialysis of a crude culture filtrate as a result of the slow diffusion of the enzymes through the cellophane membrane.

References p. 536.

When the crude culture filtrate was dialysed against an equal volume of distilled water at 4° a slow diffusion of the enzymes occurred. After 16 days the ratio of proteinase to peptidase activity inside the dialysis sac was 2.68 whilst that of the dialysate was 1.87. Thus although the peptidase passed through the membrane slightly more readily than the proteinase, the proteinase to peptidase ratio was not significantly altered. In addition, the slow rate of diffusion of both enzymes precluded the use of dialysis as a practical method for their separation.

6. *Separation of the enzymes by adsorption on heat-killed Gram-negative cells*

In Part 2 it was shown that when the Actinomyces enzyme solution is added to a suspension of heat-killed Gram-negative cells (*Bact. lactis aerogenes*), the latter acts initially as a substrate for the proteinase and this enzyme is adsorbed more rapidly by the cells. Thus it appeared that under controlled conditions, it would be possible to remove the proteinase from solution by adsorption on its insoluble substrate.

A number of experiments were carried out in an attempt to obtain a solution free from proteinase activity in this way. Equal volumes of the purified enzyme solution and a suspension of heat-killed *Bact. lactis aerogenes* cells equivalent in opacity to 5 times No. 10 on MacFarlane's barium sulphate standard scale, were mixed and incubated at 37°. With one enzyme solution in which the ratio of proteinase to peptidase (57 U/ml proteinase, 155 U/ml of peptidase) was lower than usual, it was found that the proteinase was adsorbed by the cells after 24 hrs at 37°, whereas the residual solution contained 130 U/ml of peptidase activity. In general, however, the proteinase was not completely adsorbed by the cells under the above conditions and, for more or less complete adsorption, it was necessary to centrifuge the mixture after 24 hours and to add a further suspension of heat-killed *Bact. lactis aerogenes* to the supernatant.

The procedure involving the adsorption of the proteinase on its substrate suffered from the limitations that the enzyme was not removed rapidly from the solution, and, in consequence, some lysis of the cells occurred during the period of the experiment. This resulted in the contamination of the enzyme solution with various cell constituents including products of proteolysis which formed a substrate for the peptidase. Thus, as adsorption was continued, the peptidase was removed from the solution. When this occurred the proteinase was released and after a further 24 hours' incubation, the activity of this enzyme appeared more than double that of the initial solution (Table VI) owing to the liberation of activators (*e.g.*, calcium ions, *cf.* page 531) by lysis of the cells.

TABLE VI

RESULTS OF A TYPICAL EXPERIMENT TO SHOW THE ADSORPTION OF THE PROTEOLYTIC ENZYMES ON HEAT-KILLED CELLS OF *Bact. lactis aerogenes* AT 37°

Treatment of solution	*% lysis of cells*	*Enzyme units/ml*	
		Proteinase	*Peptidase*
Initial enzyme solution	—	79	112
After 24 hrs with cells	65.5	30	94
After a further 8 hrs with fresh cells	—	8	144
After a further 24 hrs with fresh cells	52.0	36	180
24 hrs. supernatant after a further 24 hrs. incubation without cells	—	169	48

References p. 536.

Attempts to overcome the above difficulties by carrying out the adsorption at lower pH values (pH 5.0) and lower temperatures (4°) were unsuccessful. Little adsorption occurred at the lower temperature and at pH 5.0, the enzymes were deactivated.

Characterisation of the Actinomyces proteolytic enzymes

The effect of inhibitors

In Part I of this series it was shown that the lytic activity of the Actinomyces culture filtrate was inhibited by the presence of hydrogen sulphide, thioglycollic acid and formaldehyde. Further experiments on the lysis of heat-killed *Bact. lactis aerogenes* (MUGGLETON AND WEBB[2]) revealed that cysteine and glutathione had some inhibitory effect on the clearing of the cell suspension. The effect of various substances on the proteinase activity of a culture filtrate as measured viscosimetrically with a casein substrate is shown in Table VII. In these determinations the substrate solution was adjusted to pH 7.0 after the addition of the inhibitor (other than hydrogen cyanide).

TABLE VII

THE EFFECT OF VARIOUS INHIBITORS ON THE PROTEINASE ACTIVITY OF ACTINOMYCES CULTURE FILTRATE

Substance	*Molar concn in digestion mixture*	*Activity (U/ml)*
Control (No inhibitor)		71.0
Sodium thioglycollate	0.087	50.4
Cysteine	0.075	52.2
Sodium iodoacetate	0.081	70.4
Manganese sulphate	0.01	70.2
Cobalt nitrate	0.01	71.0
HCN gas	—	0

The equal inhibition of the proteinase by cysteine and sodium thioglycollate explains the reduced lytic activity of the Actinomyces filtrate in the presence of these substances. PETERS AND WAKELIN[5] showed that equimolar concentrations of compounds containing a thiol group gave equal inhibition of trypsin and chymotrypsin, and, in this respect, the Actinomyces proteinase resembles these enzymes.

Activation of the proteinase

It was known that the lytic activity of culture filtrates was considerably reduced on dialysis, and it was found that the activity could be partially restored by the addition of the concentrated dialysate to the system. Activation of the dialysed enzyme also resulted from the addition of the uninoculated medium, and was shown to be due to the presence of calcium ions. The activity of the dialysed system reached a maximum at a calcium concentration of c. 0.12 g Ca^{++}/l. Lytic activity was completely inhibited when the Ca^{++} concentration was increased to 5 times this value.

It was established that calcium had no effect on the activity of the ribonuclease of the culture filtrate. Activation of the proteinase was not apparent when Ca^{++} was added to a suspension of heat-killed *Bact. lactis aerogenes* cells, unless the latter were first exhaustively dialysed, since the cell suspension itself contained sufficient Ca^{++} to activate the enzyme.

References p. 536.

The lytic activity of an enzyme solution against a dialysed cell suspension was inhibited by the addition of just sufficient potassium oxalate to precipitate all the Ca^{++}. Furthermore, this solution, after centrifuging to remove the calcium oxalate, was without proteinase activity. Both the bacteriolytic and proteinase activities of this solution were restored by the addition of Ca^{++}. The calcium-free casein substrate used in the latter experiments was prepared by prolonged dialysis of a casein solution against repeated changes of 0.1 *M* phosphate buffer, pH 7.0, at 4°.

In connection with the effect of calcium on the activity of the Actinomyces proteinase it may be recalled that GORINI[6] has demonstrated that this ion plays an important part in the activation and stability of a number of different bacterial proteinases.

Activity of the proteolytic enzymes against synthetic peptide substrates

BERGMANN[7] has shown that proteolytic enzymes may be divided into exopeptidases (those which split a terminal amino acid from a peptide chain) and endopeptidases (those which can hydrolyse only internal bonds in the peptide chain) and that a characteristic "backbone" and "side chain" configuration is necessary for the action of a given proteolytic enzyme.

In the present work, an attempt was made to classify the two proteolytic enzymes of the Actinomyces filtrate by a study of their action on the synthetic di- and tri-peptide substrates used by BERGER, JOHNSON AND PETERSON[8].

A 0.02 *M* solution of the synthetic peptide substrate was prepared in distilled water and, where necessary, adjusted to pH 7.0. To 10 ml of this solution was added 5 ml of distilled water followed by 5 ml of the enzyme solution. Toluene (0.1 ml) was added as a preservative, and the solutions then incubated at 37°. The hydrolysis of the substrate was followed by titration of the increase in free amino groups by the acetone titration method of LINDERSTROM-LANG[9]. The percentage hydrolysis of one peptide bond in the various substrates was calculated from the increase in the amount of 0.02 *N* alcoholic hydrochloric acid required to titrate 2 ml of the digestion mixture. The results obtained with 7 synthetic peptides are summarised in Table VIII.

TABLE VIII

HYDROLYSIS OF SYNTHETIC PEPTIDES BY ACTINOMYCES CULTURE FILTRATE

Peptide substrate	*Percentage hydrolysis of one peptide bond in*				
	1 hr	*2.5 hrs*	*4 hrs*	*7 hrs*	*24 hrs*
Glycyl-glycyl-glycine	0	—	—	0	0
Glycyl-glycine	0	—	—	0	0
DL-leucyl-glycine	1	—	—	8	35
DL-leucyl-glycyl-glycine	9	24	31	47	51
D-leucyl-glycyl-glycine	0	0	0	0	0
Glycyl-L-leucine	0	0	0	0	0
DL-alanyl-glycine	0	0	0	0	0

From the results it is apparent that only the leucyl peptides of the peptides listed in Table VIII are hydrolysed by the Actinomyces culture filtrate, and that of these, DL-leucyl-glycine is hydrolysed more slowly than DL-leucyl-glycyl-glycine. Although

References p. 536.

47% hydrolysis of the latter substrate was observed after 7 hours, the hydrolysis after 24 hours only amounted to 51%. The addition of more enzyme at this stage did not result in any further hydrolysis (after 19 hours), and it was concluded that the enzyme was capable only of hydrolysing either the D or L form of the DL peptide. This was confirmed by the fact that D-leucyl-glycyl-glycine was unaffected by the enzyme (Table VIII). From these results it appears reasonable to assume that in the hydrolysis of DL-leucyl-glycine, only the L-leucyl-glycine isomer is susceptible to the enzyme.

In order to determine which of the two peptide bonds was hydrolysed when DL-leucyl-glycyl-glycine was used as a substrate, the products of the hydrolysis were identified by paper chromatography. Spots of the hydrolysis mixture were run on single dimensional chromatograms (Whatman No. 1 paper) with phenol saturated with water as solvent, together with control spots of the enzyme solution, DL-leucyl-glycyl-glycine, DL-leucyl-glycine, glycyl-glycine, glycine and DL-leucine. After 16 hours the chromatograms were dried and the positions of the amino acids and peptides revealed with ninhydrin. The results showed conclusively that the hydrolysis of DL-leucyl-glycyl-glycine resulted in the liberation of leucine and glycyl-glycine. No free glycine was liberated. The examination of the hydrolysis products of DL-leucyl-glycine in the same way showed that this peptide was hydrolysed to leucine and glycine, spots corresponding to both these amino-acids appearing on the paper.

The inability of the Actinomyces enzyme to hydrolyse DL-alanyl-glycine was of interest, since BERGER, JOHNSON AND PETERSON[8] have shown that bacterial peptidases which hydrolysed leucyl-glycyl-glycine would also hydrolyse the alanyl peptide, though, in some cases, less readily.

Since the foregoing experiments were carried out with the unfractionated culture filtrate, it was not established whether the leucyl peptides were hydrolysed by the proteinase or the peptidase, although, by analogy with other enzymes, there was presumptive evidence that the hydrolysis was due to the peptidase. Accordingly the enzyme solution (f1) was fractionated on foam as described above. Three fractions (f2, f3 and f4) were foamed over at ammonium sulphate concentrations of 0.5, 0.67 and 1.0 saturation respectively. The proteinase (casein substrate), peptidase (peptone substrate) and peptidase (leucyl-glycyl-glycine substrate) activities of these fractions, and of the solution (f5) remaining after foaming were estimated. For convenience in this experiment the activities of the solutions against leucyl-glycyl-glycine were expressed in arbitrary units, such that 100 units of activity/ml corresponded to an increase in titration of 1.0 ml of 0.02 *N* hydrochloric acid in 7 hours. The results (Table IX) show that the hydrolysis of leucyl-glycyl glycine is due to the peptidase since the ratio $\frac{\text{peptidase (peptone substrate)}}{\text{peptidase (leucyl-diglycine substr.)}}$ for the various fractions is a constant, whereas the ratio $\frac{\text{proteinase (casein substrate)}}{\text{peptidase (leucyl-diglycine substr.)}}$ shows a wide variation.

Action of the Actinomyces culture filtrate on chloracetyl-L-tyrosine

In order to exclude the presence of a carboxypeptidase in the culture filtrate, the action of the latter was studied on chloracetyl-L-tyrosine (BERGMANN AND FRUTON[10]). A solution of chloracetyl-L-tyrosine was adjusted to pH 7.2 by the dropwise addition of 0.1 *N* sodium hydroxide and diluted with water to give a 0.02 *M* solution. To 5 ml

References p. 536.

TABLE IX

PROTEINASE, PEPTIDASE AND LEUCYL-GLYCYL-GLYCINE (L-G-G) SPLITTING ACTIVITIES OF ACTINOMYCES CULTURE FILTRATE FRACTIONATED ON FOAM

Activity	*Fractions*				
	f1	*f2*	*f3*	*f4*	*f5*
Proteinase (U/ml)	96.4	97.6	44.4	26.4	0
Peptidase (U/ml)	54	80	76	40	0
Peptidase (leucyl-diglycine substrate) (U/ml)	56	80	78	46	0
Ratio $\frac{\text{Proteinase}}{\text{Peptidase (l-g-g)}}$	1.78	1.22	0.57	0.57	—
Ratio $\frac{\text{Peptidase (peptone)}}{\text{Peptidase (l-g-g)}}$	0.96	1.00	0.97	0.87	—

of this solution were added 2.5 ml of the Actinomyces culture filtrate and 2.5 ml of distilled water. The mixture was incubated at 37° and at intervals up to 24 hours, 2 ml aliquots were withdrawn and titrated with 0.01 *N* sodium hydroxide (phenol red indicator). No increase in the titration volume was recorded.

Action of the Actinomyces enzyme on benzoyl-L-arginine amide

BERGMANN[7] showed that endopeptidases (proteinases) may be characterised by their hydrolytic action on certain synthetic peptides of low molecular weight which possessed the necessary "backbone" and "side chain" groupings. Since it was apparent from the results previously recorded, that in many respects the Actinomyces proteinase was similar to trypsin, the hydrolysis of benzoyl-L-arginine amine by these enzymes was compared. It was shown by BERGMANN, FRUTON AND POLLOK[11] that benzoyl-L-arginine amide was hydrolysed rapidly by trypsin with the formation of benzoyl-L-arginine and ammonia.

Benzoyl-L-arginine amide was synthesised by essentially the method of BERGMANN *et al.*[11]. After benzoylation of the arginine, the resulting benzoyl derivative could not be induced to crystallise. The reaction mixture was therefore dried in the frozen state and the residue extracted with dry methanol. Concentration of the combined extracts afforded crystals of benzoyl-L-arginine which were converted to the amide in the usual way.

The enzymic hydrolysis of the benzoyl-L-arginine amide was followed by the titration of the liberated carboxyl groups by the alcohol titration method (GRASSMAN AND HEYDE[12]). To a 0.02 *M* solution of the substrate (5 ml) was added 0.1 *M* phosphate buffer pH 7.0 (1 ml), water (2 ml) and the Actinomyces enzyme solution (2 ml). The mixture was incubated at 37° and aliquots (2 ml) titrated at intervals up to 24 hours. In a similar experiment in which the Actinomyces enzyme was replaced by commercial trypsin, the solution of the latter enzyme was suitably diluted such that it had the same activity against casein as the Actinomyces enzyme (95 U/ml). The results (Table X) emphasize the similarity between the Actinomyces proteinase and trypsin.

It was established that the hydrolysis of benzoyl-L-arginine amide by the Actinomyces enzymes was due specifically to the proteinase since the solution of the latter enzyme alone (activity 41 U/ml), which was obtained when a solution of the proteinase and peptidase was maintained at 60° (page 527) readily hydrolysed the synthetic substrate.

References p. 536.

TABLE X

THE HYDROLYSIS OF BENZOYL-L-ARGININE AMIDE BY THE ACTINOMYCES PROTEINASE AND BY TRYPSIN

Enzyme	*Percentage hydrolysis of benzoyl-L-arginine amide*			
	1 hr	*3 hrs*	*5 hrs*	*24 hrs*
Actinomyces enzyme	13	41	54	61
Trypsin	8	24	34	42

DISCUSSION

From the results presented in the experimental section, it is apparent that the two enzymes which constitute the exocellular proteolytic enzyme system of the Actinomyces strain, are an exopeptidase (the "peptidase") and an endopeptidase (the "proteinase") respectively. That these enzymes are of similar molecular weight is shown by the fact that they are not easily separated either by fractionation with acetone, or ammonium sulphate or by dialysis.

Although the separation of the enzymes by the various methods of fractionation studied could not be accomplished without a considerable loss of activity, the selective deactivation of the more labile peptidase by heat and the separation of the proteinase on foam, permitted the unequivocal characterisation of the enzymes.

The fact that the proteinase hydrolyses benzoyl-L-arginine amide shows that the enzyme is an endopeptidase similar in action to trypsin (*cf.* BERGMANN[7]).

The peptidase is an exopeptidase, apparently similar to the leucyl peptidases of common occurrence in bacteria, plants and animals (BERGER AND JOHNSON[13]). It is markedly specific in its action in so far as it is without action on alanyl-glycine and glycyl-leucine and can only hydrolyse the peptide link between the leucine and glycine of the L-leucyl peptides when the leucine bears the unsubstituted amino group.

It would appear that the hypothesis previously advanced to explain the lysis of killed Gram negative bacteria by the Actinomyces proteolytic enzyme system is substantially correct. The initial breakdown of the protein constituents of the killed Gram negative cell, which also occurs as a result of the action of trypsin, is due to the endopeptidase. In view of the specificity of the exopeptidase however, the postulate that this enzyme hydrolyses the protein fragments resulting from the action of the endopeptidase can only be partially true, since it is evident that some of the peptides thus liberated would be resistant to the enzyme.

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References p. 536.

SUMMARY

The proteinase of the exocellular bacteriolytic system of a strain of soil Actinomyces is obtained free from peptidase activity by selective deactivation of the latter enzyme at 60°.

Separation of the two enzymes of the proteolytic system has been achieved by a combination of fractional precipitation and adsorption on foam.

A study of the action of the enzymes on synthetic peptide substrates indicates that the proteinase is an endopeptidase of the trypsin-type, whilst the peptidase is an exopeptidase which hydrolyses the leucyl-glycine bond in leucyl-peptides.

RÉSUMÉ

La protéinase du système bactériolytique d'une souche d'Actinomyces est libérée de l'activité de la peptidase par la désactivation sélective de cette enzyme a 60°.

La séparation des deux enzymes du système protéolytique a été obtenu par la combinaison d'une precipitation fractionnée et l'adsorption sur écume.

Une étude de l'action des enzymes sur des substrats peptidiques synthétiques indique que la protéinase est une endopeptidase du type trypsine, tandis que la peptidase est une exopeptidase qui hydrolyse le lien leucyl-glycine dans les leucyl-peptides.

ZUSAMMENFASSUNG

Die Proteinase, die im exocellulären bakteriolytischen System eines Stammes von Erde-Aktinomyces vorkommt, kann von der begleitenden Peptidase-Wirkung befreit werden durch die Inaktivierung der Peptidase bei 60°.

Die Trennung der beiden Enzyme des proteolytischen Systems ist durch eine Kombination von fraktionierter Präcipitation und Adsorption am Schaum erreicht worden.

Die Untersuchung der Enzymwirkung auf synthetische Peptide zeigt, dass die Proteinase eine Endopeptidase von der Art des Trypsins ist, während die Peptidase eine Exopeptidase ist, die in Leucin-Peptiden die Leucin-Glycin-Bindung hydrolysiert.

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