THE PROTEOLYTIC SYSTEM OF *BACILLUS LICHENIFORMIS*

by

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We have been studying the proteolytic enzymes of bacteria and considering their possible use in industrial processes and as analytical tools in the study of protein structure. Microbial enzymes are specially suitable for industrial use as they can be produced economically on a large scale by controlled fermentation. As analytical reagents they are of importance because, compared to plant and animal proteases, bacterial proteases are extremely large in number and even with the limited information available about them it is clear that they exhibit wide variations in range of activity, substrate specificity and mode of action. Thus MASCHMANN[1] claimed the preparation of three different proteases from aerobic and anaerobic bacteria—a protease from aerobic bacteria, acting on a variety of substrates ranging from ovalbumin to peptone and requiring no activators; another from gas gangrene organisms acting on gelatin and gluten and a third enzyme from other anaerobic organisms requiring activation by sulphydryl compounds or cyanide. A specific collagenase which acts on no protein other than gelatin and collagen has been found in some bacteria[2,3]. Evidence has been presented recently for the presence of two different proteases from *Aspergillus oryzyae*—a "viscometric protease" reducing the viscosity of gelatin very rapidly and believed to act preferentially on high molecular weight material and a "gravimetric protease" degrading more readily products of lower molecular weight[4]. There is also evidence of the existence of a multiplicity of bacterial peptidases differing in specificity and response to activators. MASCHMANN[1] inferred from his experiments that the dipeptidase of anaerobic organisms is no single enzyme but variously combined mixtures of enzymes or of apoenzymes with the same coenzyme.

One of the most interesting discoveries in the field of enzyme action in relation to protein structure was the demonstration by LINDERSTRØM-LANG AND OTTESEN[5] of the transformation of one crystalline protein to another—of ovalbumin to plakalbumin—by the action of the extracellular protease from a strain of *Bacillus subtilis*. OTTESEN AND VILLEE[6] reported that the enzyme was without di- and tripeptidase activity when tested on alanylglycine and alanylglycylglycine. CHRISTENSEN[7] from the same laboratory, however, concluded that the above enzyme preparation probably contains peptidase.

More recently a proteinase from *B. subtilis* has been obtained in pure crystalline form and its action on a number of different proteins studied[8]. TUPPY[9] reported that a preparation of the crystalline enzyme obtained from the Carlsberg Laboratory acting on the phenylalanyl chain of oxidised insulin hydrolysed most of the peptide bonds without exhibiting any selectivity.

References p. 110.

These observations on the *B. subtilis* enzyme are of special interest in connection with the present work which describes the proteolytic activity of the extracellular enzymes obtained from a closely related organism *B. licheniformis*. On screening for proteolytic activity about fifty bacteria from the culture collection in the laboratory and of new isolates, a bacterium isolated from decaying cassava tuber was found to be the source of a powerful extracellular enzyme which could be easily prepared from the culture filtrate in a cell-free condition and obtained in a stable solid form by precipitation with ethanol or acetone. In comparison with the properties of the enzyme from *B. subtilis* studied in the Carlsberg Laboratory, the enzyme system from *B. licheniformis* now described contains a protease which acts equally well on native and denatured proteins, including ovalbumin; has pronounced leucyl aminopeptidase activity; hydrolyses di- and tripeptides and acts on di-, tri- and tetraglycines. It has, however, no action on synthetic substrates considered specific for pepsin, trypsin or chymotrypsin.

EXPERIMENTAL

Materials

Casein and crystalline edestin were prepared by the usual methods. For denaturation they were heated in water at 100° for 15 minutes. The collagen used was ossein prepared from long bones of sheep which were first defatted and demineralized and treated with trypsin to remove extraneous proteins. Commercial preparations of gelatin (Gold Label) and crystalline egg albumin (Armour Laboratories) were used.

Carbobenzoxy-L-glutamyl-L-tyrosine, α-benzoyl-L-argininamide, carbobenzoxyglycyl-L-phenylalanine, carbobenzoxyglycyl-L-phenylalaninamide and L-leucinamide were synthesised according to procedures described by SMITH[10]. DL-leucylglycine, DL-leucylglycylglycine, DL-alanylglycylglycine, diglycine and triglycine were prepared according to FISCHER[11]. Tetraglycine was prepared by the polymerisation of glycine ethylester[12]. Glycyl-L-tyrosine, DL-alanyl-DL-asparagine, DL-alanyl-DL-phenylalanine and DL-alanyl-DL-methionine were obtained from Nutritional Biochemicals, New York. The purity of the peptides was established by paper chromatography.

Measurement of enzyme activities

Protein hydrolysis was determined by Sorensen's formol titration. The "test mixture" contained 10 ml of 1% casein (B.D.H. light white soluble) solution buffered at pH 7.4 and 0.1 ml of enzyme concentrate (equivalent to 1 ml of culture filtrate) in a total volume of 11 ml. After incubation at 37° for 30 minutes, 5 ml of the reaction mixture was used for titration and the results expressed in terms of ml 0.02 N NaOH. Substrate and enzyme controls were run simultaneously. Under the above conditions of estimation, activity was found to be proportional to concentration of enzyme.

Peptide hydrolysis was followed by alcoholic titration by the method of GRASSMANN AND HEYDE[13], using a Conway microburette and a magnetic flea for stirring. Amide hydrolysis was followed by estimation of the liberated ammonia by the microdiffusion method of CONWAY[14]. The optically active substrates were taken in 0.05 *M* and racemic substrates in 0.1 *M* final concentration, the digestion temperature being 37°. Amino-N and carboxyl-N were determined according to VAN SLYKE[15] and VAN SLYKE, DILLON, MACFADYEN AND HAMILTON[16] respectively and total-N by the micro-Kjeldahl method.

Paper chromatography

Two solvent systems, phenol saturated with water, in the presence of 0.3% ammonia and cyanide and the alcohol phase of freshly prepared *n*-butanol-acetic acid-water (4:1:4)[17], were generally used. One-dimensional chromatography was carried out by the ascending technique with Whatman No. 1 paper and an irrigation time of six to seven hours. After the run the papers were air dried and spots developed by passing through a 0.2% solution of ninhydrin in acetone. Two-dimensional chromatography was also carried out on casein digests with the same pair of solvents using a frame similar to that of DUTTA, DENT AND HARRIS[18].

Ion exchange column analysis

The procedure described by MOORE AND STEIN[19] was followed using Dowex 50, 12% crosslinked, 200-400 mesh resin. The identity of the elution peaks was determined from their positions with reference to a run with a mixture of known amino acids and also by desalting the eluate fractions and identifying the amino acids by one-dimensional paper chromatography. The desalting of the acidic and neutral amino acids was carried out in a manner similar to that described by STEIN[20]

References p. 110.

but using Deacidite FF (Permutit Company, England) instead of Dowex-2. With this resin, a greater volume of acid was found necessary for elution of some of the adsorbed amino acids; hence twice the volume recommended by STEIN was used.

Isolation of the organism

The bacterium from decaying cassava was isolated in pure state by repeated dilution plating and was maintained on nutrient agar. Morphological and nutritional characteristics studied, indicated that the organism belonged to the *B. subtilis* group. It was subsequently identified as *B. licheniformis* (Weigman) Gibson by Dr. RUTH E. GORDON of Rutgers University. Compared to the standard strain of *B. subtilis* (NCIM 2006) the organism isolated from cassava has a 30 % higher proteolytic activity.

Preparation of protease concentrate

Enzyme production was investigated when the organism was grown on several media: nutrient broth containing 1 % glucose and a basal medium containing 1 % glucose, 0.05 % magnesium sulphate 0.1 % potassium dihydrogen phosphate and 0.15 % calcium chloride to which 2 % (w/v) of whole milk powder, groundnut meal (powdered oilcake), ground lentils or casein was added as supplement. The culture filtrate from the medium containing groundnut meal gave the highest yield of proteolytic activity, which was usually 25 % higher than that obtained in peptone broth cultures. The results recorded in this paper refer to the enzyme formed by the organism when grown on the medium containing salts and groundnut meal. Results obtained with the glucose-nutrient broth medium were qualitatively similar.

Fermentation was carried out at 30°–31° under submerged conditions in 500 ml conical flasks each containing 150 ml of the medium and agitated on a rotary shaker. Each flask was inoculated with 5 ml of a 24-hour-old shake culture grown on nutrient broth. At the end of four days of incubation when proteolytic activity was found to reach a maximum, the culture fluid was centrifuged and the centrifugate filtered through a Seitz filter. The filtrate was concentrated to approximately a tenth of the original volume by distillation *in vacuo* at a bath temperature of 30°. It was repeatedly found that by this procedure 95 % of the total original activity was recoverable. The concentrate was dialysed against running deionised water for about 15 hours at 15° to remove most of the salts and protein degradation products. The dialysed preparation stored under toluene in the cold was found to retain its activity unimpaired for several weeks. The enzyme could be obtained as a solid preparation by precipitation in the cold with two volumes of acetone or alcohol with a recovery of 90–95 % of the original activity. Tested on casein as substrate this preparation showed on the average a 25 % higher activity than a commercial trypsin preparation (B. D. H.).

RESULTS AND DISCUSSION

Proteinase activity

(a) *Optimum pH:* The pH optimum was determined using citrate-HCl, acetate, phosphate and borate buffers to cover the pH range 2–10. Reaction was allowed to take place in 6.0 *N* urea solution to prevent precipitation of casein at the lower pH ranges. The results presented in Fig. 1 show a sharp optimum at pH 7.4 with rapid fall in the rate of hydrolysis on either side of the optimum; in this respect the enzyme differs from the *B. subtilis* enzyme[8] which has an optimal pH at 10–11.

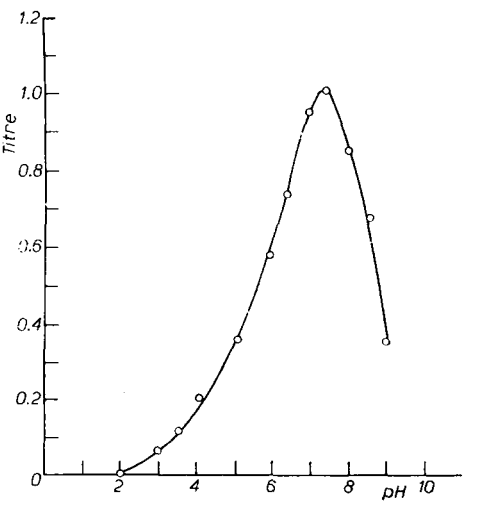

Fig. 1. Effect of pH on proteinase activity.

(b) *Effect of cysteine, cyanide, iodoacetate and metal ions.* Cysteine has no activating or inhibiting effect on this proteinase in contrast to the inhibition observed on the proteolytic acitivity of enzymes of aerobic organisms studied by MASCHMANN[21], WEIL, KOCHOLATY AND SMITH[22], and of the activation observed with anaerobic organisms[23,24,25].

References p. 110.

Cyanide and iodoacetate were also without effect. These observations would indicate that the enzyme under investigation does not contain any active sulphydryl group[26]. In contrast to the stabilising and activating influence of some bivalent cations reported on certain proteolytic enzymes[24, 27, 28], manganese, cobalt, mercury and copper showed inhibition in ascending order. Similar results have been recorded[8] for the *B. subtilis* enzyme, NaCN, ICH_2COONa and $HgCl_2$ showing no appreciable change in activity.

TABLE I

EFFECT OF CYSTEINE, IODOACETATE AND METAL IONS ON PROTEINASE ACTIVITY

0.1 ml of the enzyme concentrate was incubated with the indicated substance and buffer at pH 7.4 for 2 hours at 37°. Activity was measured as usual with casein as substrate.

Substance	*Molarity*	*Titre (ml 0.02 N-Sodium hydroxide)*
None	—	0.70
Cysteine	0.01	0.70
Iodoacetate	0.005	0.69
Iodoacetate	0.01	0.70
Potassium cyanide	0.01	0.67
Manganese sulphate ($MnSO_4, 4H_2O$)	0.01	0.60
Cobalt chloride ($CoCl_2, 6H_2O$)	0.02	0.60
Mercuric sulphate ($HgSO_4, 2H_2O$)	0.01	0.54
Cupric chloride ($CuCl_2, 2H_2O$)	0.01	0.51

TABLE II

PROTEIN HYDROLYSIS

Proteins in 2% (w/v) solutions at pH 7.4 incubated with enzyme (protein equivalent being 5% of substrates); enzyme action stopped at end of digestion periods by heating in boiling water bath for 10 minutes. The values are expressed as % amino-N (also as carboxyl-N for casein) in terms of total-N.

Protein		*Hydrolysis %*					
		1 day	*2 days*	*3 days*	*4 days*	*5 days*	*7 days*
Casein	Amino-N	14.4	21.4	26.3	31.2	35.3	43.9
	Carboxyl-N	5.62	8.26	17.35	20.30	26.35	30.5
Egg albumin	Native	8.8	19.8	24.5	30.0	34.0	36.5
	Denatured	13.4	20.5	25.4	31.1	31.4	31.4
Edestin	Native	10.8	18.6	27.9	32.0	34.2	35.0
	Denatured	19.8	27.4	34.2	35.0	35.2	35.5
Gelatin		12.8	14.0	15.1	17.6	17.8	18.1

(c) *Action on proteins.* Table II gives the extent of hydrolysis of different proteins in the course of 1–7 days of enzyme action. Hydrolysis is far reaching with most proteins, amino-N liberated reaching with casein 64% of total amino-N obtained on complete hydrolysis by acid, 47% with egg albumin and 58% with edestin. With egg albumin and edestin the native or crystalline protein was acted on to the same extent as the denatured proteins. The limited hydrolysis noted with the derived protein, gelatin, is not easy to

References p. 110.

interpret, as the low amino-N observed might have been due either to a preponderance of imino-N or to limited hydrolysis caused by the presence of enzyme resistant peptide bonds containing imino acids[29]. The hydrolysis of ossein was not studied quantitatively; its dissolution by the *B. licheniformis* enzyme took place in 70 hours in comparison to 20 hours required by pepsin; trypsin showed no detectable action in 7 days.

TABLE III

SYNTHETIC SUBSTRATES FOR ENDOPEPTIDASES

The digestion mixture contained 0.05 *M* substrate and 0.1 ml enzyme in a total volume of 2 ml at pH 7.4. Cysteine and KCN were added to give final concentrations of 0.01 *M* and 0.02 *M* respectively. Titre values are expressed in terms of 0.01 *N* KOH in the case of carbobenzoxy-L-glutamyl-L-tyrosine and 0.015 *N* NaOH in the case of amide substrates.

Substrate	*Titre, ml*			
	0 h	*24 h*	*48 h*	*72 h*
Carbobenzoxy-L-glutamyl-L-tyrosine	1.89	1.89	1.89	1.90
Carbobenzoxy-L-glutamyl-L-tyrosine + cysteine	1.94	1.94	1.94	1.95
Carbobenzoxy-L-glutamyl-L-tyrosine + cyanide	1.91	1.91	1.91	1.91
α-benzoyl-L-argininamide	0.954	0.960	0.960	0.960
α-benzoyl-L-argininamide + cysteine	1.050	1.050	1.050	1.050
α-benzoyl-L-argininamide + cyanide	1.020	1.020	1.020	1.020
Carbobenzoxyglycyl-L-phenylalaninamide	1.005	1.005	1.006	1.006
Carbobenzoxyglycyl-L-phenylalaninamide + cysteine	1.010	1.010	1.010	1.010
Carbobenzoxyglycyl-L-phenylalaninamide + cyanide	1.010	1.010	1.010	1.010

The formation of free amino acids in appreciable quantities is shown in the case of casein by the Van Slyke carboxyl-N values, the ratio of free amino acids to amino-N increasing from 40% in 24 hours to 70% at the end of 7 days (Table II). Paper chromatography showed distinct spots for amino acids as early as 6 hours, particularly strongly at glutamic acid and leucine levels. Digests of all other proteins studied gave (Fig. 2) at the end of a digestion period of 120 hours distinct spots corresponding to the following amino acids: aspartic and glutamic acids, serine, glycine, alanine, tyrosine and leucines. That such chromatograms did not provide conclusive evidence of the presence of free amino acids became obvious when the spots were submitted to elution, hydrolysis and rechromatography according to

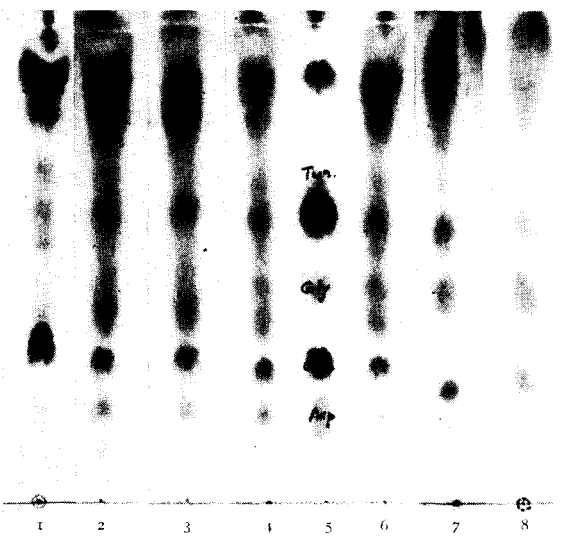

Fig. 2. Protein hydrolysis; one way chromatography, ascending technique; solvent: phenol-water (0.3% NH_3 and HCN); duration of run 7–8 hours; spots developed with ninhydrin (0.2% w/v) in acetone. Chromatograms of 120 hours digests of proteins: 1, casein; 2, crystalline egg albumin; 3, denatured egg albumin; 4, crystalline edestin; 5, reference mixture of amino acids* (bottom to top) Asp, Glu, Gly, Ala, Tyr and Leu; 6, denatured edestin; 7, ossein; 8, gelatin.

* Abbreviations for amino acids and peptides according to SANGER AND TUPPY[38].

References p. 110.

CONSDEN, GORDON AND MARTIN[30], when spots were obtained not only for the amino acid originally indicated but also for others in varying concentrations suggesting the presence of peptides occupying the same position as the amino acid on the original chromatogram. Analysis on Dowex-50 columns gave more unequivocal proof of the presence of amino acids. In Fig. 3, identification of peaks 3 to 9 and 12 to 14 by desalting and paper chromatography showed the presence of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, isoleucine, leucine and phenylalanine

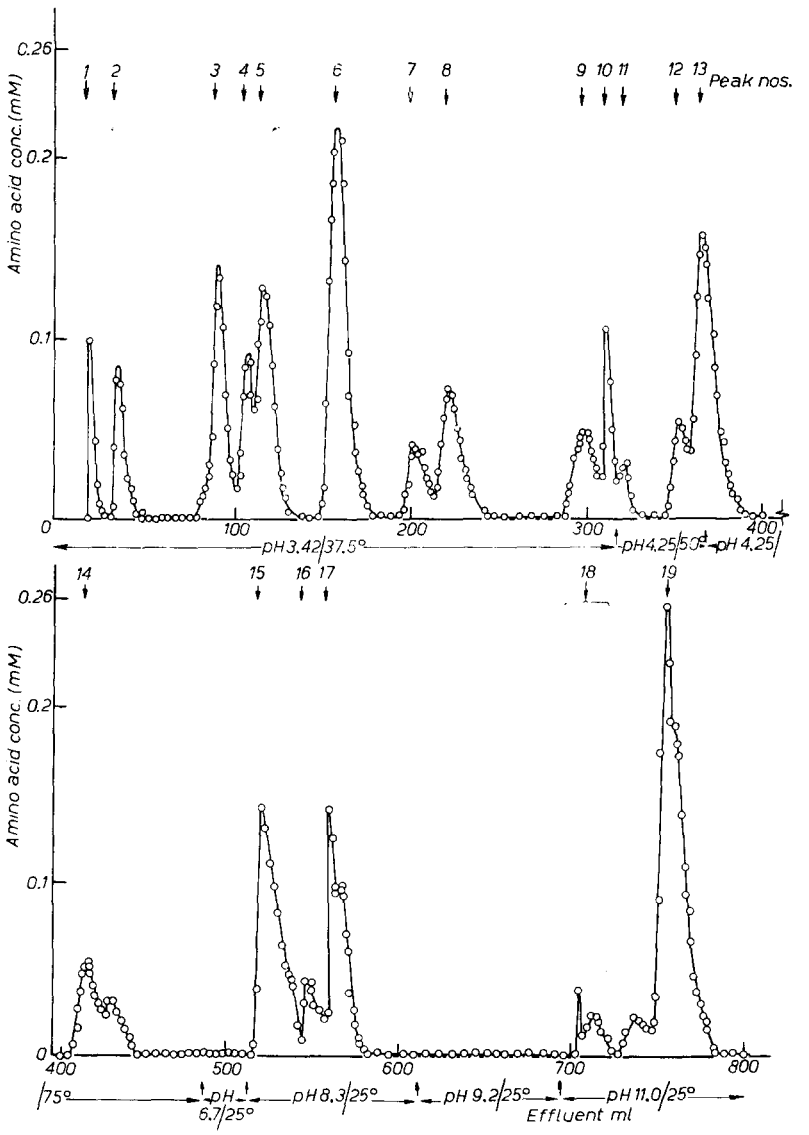

Fig. 3. Ion exchange column analysis of 168 hours digest of casein. The sample was treated with 3 volumes of hot alcohol to remove undigested protein and large peptides. The column of Dowex-50 × 12, 1.2 × 100 cm, was operated in the sodium form employing as eluants, buffers of pH and at temperatures indicated.

References p. 110.

respectively. Peaks 1, 2, 10 and 11 do not correspond to any known amino acid and are probably peptides. Two dimensional paper chromatography of these digests also showed certain spots which disappeared on acid hydrolysis of the digests and are to be attributed to peptides. Basic fractions represented by peaks 15 to 19 have not been definitely identified.

TABLE IV

L-LEUCINAMIDE

L-leucinamide, 0.05 *M*; 0.1 ml enzyme in a total volume of 2.0 ml, pH 7.2. Enzyme was incubated with the metal ion activators or inhibitors at pH 7.2 for a period of 3 hours at 37° before addition of substrate.

Substance added	*Molarity*	*Hydrolysis %*		
		2 h	*6 h*	*24 h*
None		49.0	73.0	99.0
Manganese sulphate ($MnSO_4, 4H_2O$)	0.01	49.0	74.0	99.5
Magnesium chloride ($MgCl_2, 6H_2O$)	0.01	51.0	75.0	99.5
Zinc sulphate ($ZnSO_4, 7H_2O$)	0.01	52.0	75.0	99.5
Cysteine	0.01	17.0	18.0	25.0
Cyanide	0.02	10.0	14.0	25.0

(d) *Action on peptide substrates for endopeptidases.* The enzyme showed no action on carbobenzoxy-L-glutamyl-L-tyrosine, α-benzoyl-L-argininamide, carbobenzoxyglycyl-L-phenylalaninamide, the specific substrates for pepsin, trypsin and chymotrypsin respectively (Table III). There was no hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine at pH 4.0 found to be optimum for pepsin acting on the synthetic substrate[31]. Addition of cysteine or cyanide found to activate cathepsin II[32] and *Clostridium histolyticum* protease[25] made no difference. Thus the *B. licheniformis* enzyme appears to fall in the category of the proteolytic systems of nonbacterial origin described by several authors[33,34,35] which show pronounced action on proteins but do not hydrolyse the synthetic substrates considered to be specific for endopeptidases.

TABLE V

DIPEPTIDES

Racemic peptides in 0.1 *M*, glycyl-L-tyrosine in 0.05 *M* final concentrations, 0.1 ml enzyme in total volume of 2.0 ml, pH 7.6.

Substrate	*Hydrolysis %*					
	2 h	*6 h*	*24 h*	*48 h*	*72 h*	*120 h*
DL-Leucylglycine	18.5	35.1	77.4	99.2	99.2	—
Glycyl-L-tyrosine	4.4	12.5	17.5	—	26.7	34.6
DL-Alanyl-DL-phenylalanine	0.0	0.0	0.0	0.0	0.0	—
DL-Alanyl-DL-asparagine	0.0	0.0	0.0	0.0	0.0	—
DL-Alanyl-DL-methionine	0.0	0.0	0.0	0.0	0.0	—

Peptidase activity

(a) *Optimum pH*: L-Leucinamide and DL-leucylglycine were used as substrates for determining the optimum pH for peptidase action. Acetate, phosphate and borate

References p. 110.

buffers were used to cover the pH range from 4 to 10. The results are represented in Fig. 4. The optimum pH for hydrolysis of L-leucinamide was 7.2 and of DL-leucylglycine 7.6.

(b) *Action on* L-*leucinamide*: Results in Table IV show that there is complete hydrolysis of L-leucinamide in 24 hours. Activity was not enhanced by the known metal activators of leucyl aminopeptidase[10] obviously because the enzyme was used without purification. However, the known inhibitors of this peptidase, cysteine and cyanide showed a 74% inhibition of activity in 24 hours. The picture obtained by paper chromatographic analysis of the digest showed gradual disappearance of L-leucinamide and appearance of leucine, only leucine being visible at the end of 24 hours. On the contrary in the presence of inhibitors there were prominent spots of L-leucinamide and only weak spots of leucine in the same period.

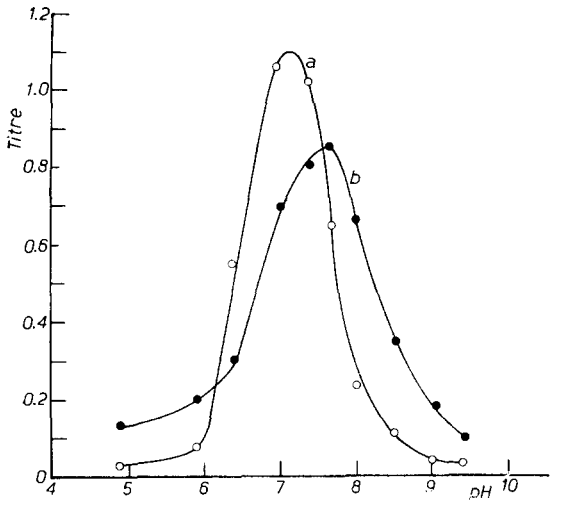

Fig. 4. Effect of pH on peptidase activity; L-Leu·NH_2 of 0.05 *M* and DL-Leu·Gly, 0.1 *M* final concentration; 0.1 ml of enzyme in total volume of 2.0 ml; incubation time 6 hours at 37°; curve (a): L-Leu·NH_2; curve (b): DL-Leu·Gly.

(c) *Action on carbobenzoxyglycyl-*L-*phenylalanine*. Under the experimental conditions described for other peptides, no measurable hydrolysis of this peptide occurred, even at the end of 168 hours. No phenylalanine spot could be found by paper chromatographic analysis of the reaction mixture up to 168 hours confirming the absence of any carboxypeptidase activity in the enzyme system.

TABLE VI

TRIPEPTIDES

Conditions same as in Table V. Cyanide was used in a final concentration of 0.02 *M*.

Substrate	*Hydrolysis %**			
	2 h	*6 h*	*24 h*	*48 h*
DL-Leucylglycylglycine	36.0	73.7	102.5	108.0
DL-Leucylglycylglycine + cyanide	3.0	12.5	23.0	30.0
DL-Alanylglycylglycine	33.0	60.5	89.0	102.5
DL-Alanylglycylglycine + cyanide	3.7	12.0	20.0	32.0

* 100% denotes complete hydrolysis of one peptide bond.

(d) *Action on dipeptides*. Results in Table V indicate that while leucyl and glycyl peptides are hydrolysed, the enzyme has no action on alanyl peptides. DL-leucylglycine is split rapidly and to completion unlike glycyl-L-tyrosine which is hydrolysed only to 34.6% at the end of 120 hours. Fig. 5, strips 1 to 5, shows the results of paper chromatographic analysis of the action of the bacterial enzyme on dipeptides.

(e) *Action on tripeptides*. From Table VI and Fig. 5, strips 6 to 11, it will be seen that both the tripeptides studied were hydrolysed by the bacterial enzyme at a fairly

References p. 110.

TABLE VII

DI-, TRI- AND TETRAGLYCINES

Conditions same as in Table V. Cobalt chloride ($CoCl_2, 6H_2O$) used in 0.001 *M* final concentration.

Substrate	*Hydrolysis %**		
	24 h	*66 h*	*96 h*
Diglycine	0.0	0.0	—
Diglycine + Co^{++}	14.0	35.0	42.0
Chloracetyldiglycine + Co^{++}	0.0	0.0	0.0
Triglycine	15.0	40.0	55.0
Triglycine + Co^{++}	93.0	113.0	120.0
Tetraglycine	13.0	35.0	44.5
Tetraglycine + Co^{++}	82.0	137.0	160.0

* 100 % denotes complete hydrolysis of one peptide bond.

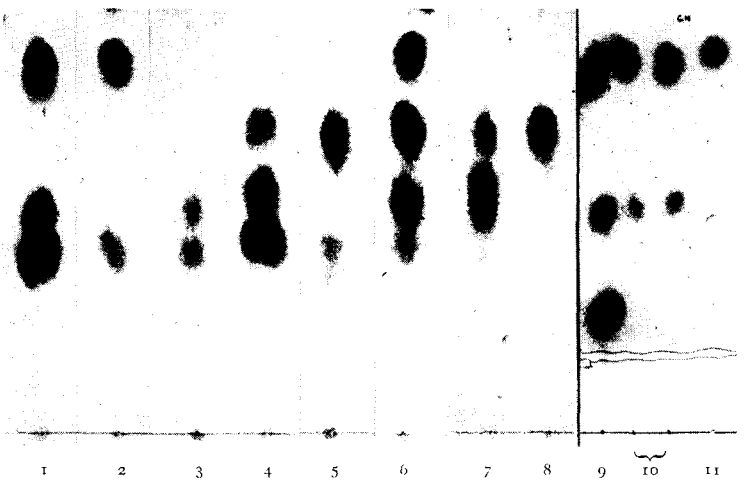

Fig. 5. Peptide hydrolysis; conditions same as in Fig. 2 except that strips 9–11, were run for 20–22 hours; identification of spots given in order from bottom to top. 1, reference mixture of Gly, Gly·Gly and Leu·Gly; 2, digest of Leu·Gly showing released Gly; the released Leu under the conditions of separation used occupies the same position as Leu·Gly; 3, Gly·Gly digest in presence of Co^{++} showing release of Gly; 4, reference mixture of Gly, Gly·Gly, Gly·Tyr and Tyr; Tyr occupies the same position as Gly·Tyr; 5, digest of Gly·Tyr showing released Gly; 6, reference mixture of Gly, Gly·Gly, Ala·Gly·Gly and Leu; 7, Ala·Gly·Gly digest showing released Gly·Gly, while Ala occupies the same position as substrates; the release of Ala confirmed by use of *n*-butanol-aqueous acetic acid solvent; 8, digestion of Ala·Gly·Gly in presence of KCN, exhibiting marked inhibition of hydrolysis; 9, reference mixture of Gly, Gly·Gly and Leu·Gly·Gly; 10, digest of Leu·Gly·Gly showing split Gly·Gly; the Leu confirmed by irrigation with *n*-butanol–aqueous acetic acid solvent; 11, Leu.

rapid rate, the hydrolysis being inhibited by cyanide to the extent of 70–79 % in 24 hours. BERGMANN AND FRUTON[36] have recorded a similar inhibition with the ereptic enzymes of the intestinal mucosa. Paper chromatographic analysis of the digests also show the mode of action of the enzyme on the two tripeptides. The action starts from the amino end of the peptides releasing leucine and glycylglycine from leucyldiglycine and alanine and glycylglycine from alanyldiglycine. On prolonged incubation the glycylglycine formed in the first stage shows some hydrolysis giving rise to a weak spot for glycine on the chromatogram.

References p. 110.

(f) *Action on di-, tri- and tetraglycines.* With diglycine 42% hydrolysis was obtained in the presence of Co^{++} ions whereas no hydrolysis was detectable upto 72 hours in the absence of the metal ion. Tri- and tetraglycines were hydrolysed to the extent of 55% and 44.5% respectively at the end of 96 hours. In both cases the enzyme was strongly activated by Co^{++} ions, hydrolysis rising to 93% and 82% respectively in 24 hours (Table VII). Chromatograms (Fig. 6) show a steady increase in concentration of diglycine and glycine up to the time of the complete disappearance of tri- and tetraglycines in 66 hours of digestion. This fact taken in conjunction with the great activation of hydrolysis of the tri- and tetraglycines as compared to diglycine show clearly that the activating influence of Co^{++} ions is not confined to dipeptide hydrolysis as usually assumed[37].

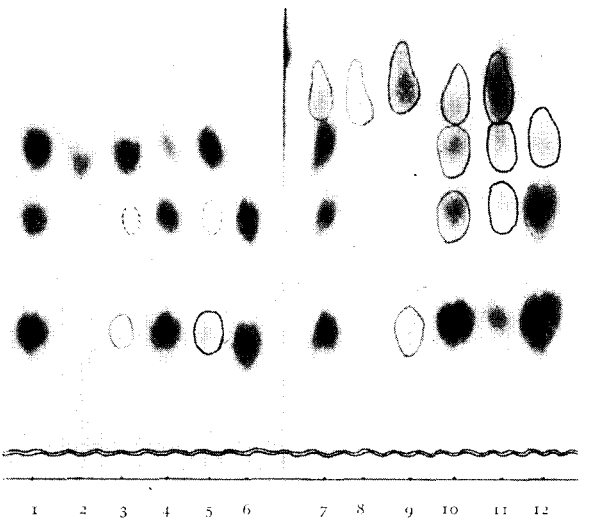

Fig. 6. Hydrolysis of tri- and tetraglycines; conditions same as in Fig. 2 except that duration of run was 20–22 hours; identity of spots from bottom to top: 1, reference mixture of Gly, Gly·Gly and Gly·Gly·Gly; 2 to 6, digests of triglycine: 2, at 0 hours; 3, at 24 hours showing small release of Gly and trace of Gly·Gly; 4, at 24 hours with Co^{++}, strong spots for Gly and Gly·Gly and weak spots for substrates demonstrating rapid hydrolysis; 5, at 66 hours without Co^{++}, feeble spots for Gly and Gly·Gly showing continued slow hydrolysis; 6, at 66 hours with Co^{++} showing complete disappearance of substrate spot and intense spots for Gly and Gly·Gly; 7, reference mixture of Gly, Gly·Gly, Gly·Gly·Gly and Gly·Gly·Gly·Gly; 8 to 12, digests of tetraglycine: 8, at 0 hours; 9, at 24 hours showing small release of Gly; 10, at 24 hours with Co^{++} showing rapid digestion with release of Gly·Gly and Gly·Gly·Gly, the large release of Gly being marked; 11, at 66 hours showing continued slow hydrolysis, the Gly spots and feeble spots for Gly·Gly and Gly·Gly·Gly being discernible; 12, at 66 hours with Co^{++} showing intense spots for Gly and Gly·Gly, feeble spot for Gly·Gly·Gly and absence of spot for substrate.

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References p. 110.

SUMMARY

1. A bacterium isolated from decaying cassava tubers and identified as *B. licheniformis* (Weigman) Gibson, produces a powerful protease in the extracellular fluid. The proteolytic activity is highest when the organism is grown on a medium containing groundnut meal.

2. The culture fluid when concentrated, dialysed and precipitated with acetone or ethanol gives a stable solid with an activity higher than that of the usual commercial trypsin.

3. The enzyme system consists of a proteinase and one or more peptidases. It acts on all proteins tried, in both native and denatured condition, with liberation of a high proportion of free amino acids, but has no action on the synthetic substrates for pepsin, trypsin and chymotrypsin. The action on proteins is neither inhibited nor activated by cysteine, cyanide or iodoacetate but is slightly inhibited by bivalent metals.

4. The peptidases present hydrolyse di-, tri- and tetrapeptides, hydrolysis starting from the amino end. According to accepted ideas leucine aminopeptidase is present, leucinamide and leucylglycine but not alanyldipeptides being hydrolysed. This hydrolysis is inhibited by cysteine and cyanide.

5. Tripeptides with leucine or alanine at the amino end are also acted upon; cyanide is a strong inhibitor of this hydrolysis.

6. Di-, tri- and tetraglycines are hydrolysed, the action on all these peptides being strongly accelerated by Co^{++} ions.

RÉSUMÉ

1. Une bactérie isolée de tubercules pourrissant de manioc et identifiée avec *B. licheniformis* (Weigman) Gibson, libère une protéase puissante dans le liquide extracellulaire. L'activité protéolytique la plus élevée est obtenue quand la bactérie est cultivée sur un milieu renfermant de la farine d'arachide.

2. Le milieu de culture concentré, dialysé et précipité par l'acétone ou l'éthanol donne un produit stable dont l'activité est plus élevée que celle de la trypsine commerciale usuelle.

3. Le système enzymatique est constitué d'une protéinase et d'une ou plusieurs peptidases. Il est actif sur toutes les protéines essayées, natives ou dénaturées, et libère une proportion élevée d'amino-acides libres, mais il est sans action sur les substrats synthétiques de la pepsine, de la trypsine et de la chymotrypsine. L'action sur les protéines n'est ni inhibée, ni activée par la cystéine, le cyanure ou l'iodoacétate mais elle est légèrement inhibée par les métaux bivalents.

4. Les peptidases présentes hydrolysent les di-, tri-, et tétrapeptides, l'hydrolyse commençant à l'extrémité aminée. Selon les conceptions actuelles, la préparation renferme une leucine aminopeptidase, puisque la leucinamide et la leucylglycocolle mais non les alanyldipeptides sont hydrolysés. Cette hydrolyse est inhibée par la cystéine et le cyanure.

5. Des tripeptides possédant une leucine ou une alanine à leur extrémité aminée sont également susceptibles; le cyanure inhibe fortement leur hydrolyse.

6. Les di-, tri- et tétraglycocolles sont hydrolysées, leur hydrolyse étant fortement activée par les ions Co^{++}.

ZUSAMMENFASSUNG

1. Ein aus faulender Cassava tubera gewonnenes Bakterium wurde als *B. licheniformis* (Weigman) Gibson identifiziert und bildet eine äusserst wirksame Protease, welche in die extrazelluläre Lösung ausgeschieden wird. Die proteolytische Wirksamkeit ist am stärksten, wenn die Bakterien auf einem erdnussmehlhaltigen Nährboden gezüchtet werden.

2. Aus der Nährlösung kann durch Einengung, Dialyse und Fällung mittels Aceton oder Ethylalkohol ein haltbarer Trockenrückstand gewonnen werden, welcher wirksamer ist, als gewöhnlich käufliches Trypsin.

3. Der Enzymkomplex besteht aus einer Protease und einer oder mehreren Peptidasen. Er erwies sich gegenüber nativen oder denaturierten Eiweisskörpern in allen untersuchten Fällen als wirksam und bildete einen hohen Prozentsatz freier Aminosäuren. Gegenüber den synthetischen Substraten des Pepsins, des Trypsins und des Chymotrypsins erwies er sich als unwirksam. Die Wirksamkeit auf Proteine wird durch Cystein, Cyanid oder Jodacetat weder gehemmt noch gefördert, doch besteht eine geringfügige Hemmung durch zweiwertige Metallionen.

4. Die im Komplex anwesenden Peptidasen spalten Di-, Tri-, und Tetrapeptide, wobei die Hydrolyse am Aminoende einsetzt. Nach allgemein üblichen Vorstellungen wird die Gegenwart von Leucinaminopeptidase angenommen, da Leucinamid und Leucylglycin, nicht aber Alanyldipeptide gespalten werden. Diese Spaltung wird durch Cystein und Cyanid gehemmt.

References p. 110.

5. Tripeptide welche Leucin oder Alanin am Aminoende enthalten, werden ebenfalls gespalten. Cyanid wirkt gegenüber dieser Hydrolyse stark hemmend.

6. Di-, Tri- und Tetraglycin werden gespalten; die Spaltung dieser Peptide wird durch Co^{++} Ionen stark beschleunigt.

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