XXXII. THE PROTEOLYTIC ENZYMES OF GREEN MALT.

II. ACTION OF THE PROTEINASE ON EGG-ALBUMIN, CASEINOGEN, EDESTIN AND FIBRIN AT DIFFERENT REACTIONS.

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DEFINITE progress in the direction of clarifying our ideas on the nature of the proteolytic enzymes of green malt has been made in the past two years, during which no less than six papers appeared [Mill and Linderstrøm-Lang, 1929; Lüers and Malsch, 1929; Hopkins, 1929; Linderstrøm-Lang and Sato, 1929, 1, 2; Hopkins and Burns, 1930]. In their main conclusions these authors agreed that green malt contains (1) a protease or proteinase (to adopt the nomenclature of Grassmann) which appears to attack edestin, gelatin, crystalline egg-albumin and Witte peptone, and (2) at least one peptidase which attacks the dipeptide leucylglycine. According to Linderstrøm-Lang and Sato [1929, 2] there is a second peptidase present, which attacks alanyl-glycine and, unlike the previous one, is not inhibited by phosphates. The present communication is concerned with the activities of the proteinase.

It seems desirable, in investigations of the relations between enzyme activity and p_H , to use as purified a preparation of the enzyme as possible. Certain difficulties however present themselves. The yield of proteinase in the preparations obtained by the adsorption and elution methods so far tried is rather low, and the quantity of mineral salts present in the elutions is sufficient to influence the properties of the substrates. For instance, it was shown by Michaelis and Szent-Györgyi [1920] that the ions of certain salts influence the p_H of maximum precipitation of caseinogen. This is in agreement with certain observations of Hardy in connection with globulins. The present authors have also observed that phosphates and citrates, the former of which are used for elution of the enzyme, and both of which are used as buffers to stabilise p_H , inhibit the activity of the proteinase at p_H 4·7, but whether this is due to a cause such as that observed by Michaelis and Szent-Györgyi or to some other inhibitive effect was not investigated. For the present, the activity of the proteinase has been investigated using ordinary cold aqueous extract of green malt as the active agent, and in the absence of added buffers except in Exp. 1. The main object of the work was to ascertain

if the proteinase attacks its protein substrates optimally at their isoelectric points, *i.e.* is specific for the zwitterions, as suggested by Willstätter, Grassmann and Ambros [1926], in the cases of papain and bromelin.

In allowing the enzyme to act at 40° or thereabouts, it is necessary to adopt a short time of action, since the enzyme, in the presence of malt extract alone, is susceptible to fairly rapid inactivation or destruction when maintained at this temperature at a reaction outside the range of p_H 3.8 to 6.0. This was investigated by Hopkins and Burns [1930], who preserved an aqueous extract of green malt with toluene at various reactions at 40°. It was found that the proteinase is destroyed in the presence of free alkali (hydroxyl ions) and of excessive acidity, its optimum stability being at p_H 4.4 to 5.0, or near the isoelectric point (presumably) of the malt extract proteins. The activity was reduced to one-half in 24 hours at p_H 6.0, and in 48 hours at p_H 3.8. In order to avoid any displacement of the p_H of the optimum activity, and any serious distortion of the p_H -activity curves, the experiments recorded here were performed by allowing the enzyme to act on the substrates over a range of p_H for 8 hours. Except in the case of edestin, the proteolysis was measured by the amount of protein rendered soluble or non-coagulable, as the case may be, at its isoelectric point. This eliminated the measurement of any action due to the peptidases. The results may be briefly stated here.

Egg-albumin. It was found that, at p_H 4.6 and 46°, proteolysis was proportional to time up to 6 hours or more (Exp. 1). The optimum activity was exhibited at p_H 3.3 in the presence of phosphate-citrate buffers (Exp. 2) and at a point more acid than p_H 3.8 in the absence of added buffers (Exp. 3). The isoelectric point of egg-albumin is at p_H 4.8 according to Sørensen. The activity at p_H 3.87 in the absence of added buffers was 1.23 times that at p_H 4.8 (Fig. 1).

Caseinogen. At 46° and p_H 5.1 proteolysis was proportional to time up to 9 hours (Exp. 5). Two optima of activity were observed at p_H 3.35 and 5.57, *i.e.* on each side of the isoelectric point, which is 4.62 according to Michaelis and Pechstein [1912] (Exps. 6 and 7).

Edestin. The relation between proteolysis and p_H for edestin was investigated by Mill and Linderstrøm-Lang [1929] who found the optimum to be at p_H 4.3. This was obtained in the presence of chlorine ions, the protein being in solution in hydrochloric acid or sodium chloride. These results have been confirmed using a shorter period of time for the reaction (Exp. 9). In view of the work of Michaelis and Szent-Györgyi [1920] already referred to, the experiment was repeated using acetic acid instead of hydrochloric acid (Exp. 10). The point of maximum precipitation of edestin is unaffected by sodium acetate, whereas in the presence of sodium chloride it is shifted in the acid direction. It was found, however, that proteolysis increased with acidity up to the maximum attainable with acetic acid as the sole acid present.

Fibrin. At 46° and p_H 4.8 proteolysis was proportional to time for 2 hours only (Exp. 12). Two optima of activity were observed at p_H 3.8 and 6.0, *i.e.*

on each side of the zone of maximum precipitation of the protein under the conditions obtaining. The isoelectric point of fibrin is generally stated to be about 6.8, for which reason fibrin was used. It was very difficult to dissolve, and, after solution had been effected under somewhat drastic conditions, the zone of maximum precipitation was at about p_H 4.8.

Ordinates. Proteolysis as cc. $N/10$ HCl.

Abcissae. p_H .

The broken lines represent the forms that the curves would presumably take, if the substrates did not flocculate within the zones of minimum solubility.

Readings from Table II are represented by crosses on the egg-albumin curve, readings from Table III (after reduction to appropriate values of the ordinate) are represented by encircled dots.

The diagram illustrates the results, ordinates representing proteolysis, the abscissae p_H .

DISCUSSION OF THE RESULTS.

The p_H optima of proteinase activity under the conditions adopted do not coincide with the isoelectric points of the respective substrates. In the cases of caseinogen and fibrin, the protein being almost insoluble over a zone of p_H including the isoelectric point, it is only to be expected that proteolysis

would be less vigorous here, since the substrate is partly or wholly in suspension, and the concentration in solution is low enough to limit the rate of enzyme action. This cannot be avoided, since, if the very small fraction of protein which remains in solution at the point of maximum precipitation were alone used as substrate, and proteolysis measured by the Willstätter or formaldehyde titration (which would be necessary in this case), we should probably be using neither caseinogen nor fibrin as substrate, but a small fraction of abnormal and possibly contaminated material. The p_H activity curves of both caseinogen and fibrin strongly suggest that, but for the insolubility of the substrate within this zone, the proteolytic action would have increased to an optimum within the zone, and in the case of caseinogen this would have approximated to the isoelectric point. However, egg-albumin, which is soluble at all reactions investigated, gives results indicating that the optimum is not at the isoelectric point (4.8) but at p_H 3.3 to 3.6. Edestin, although held in solution fairly completely by sodium chloride or acetate at all reactions investigated, showed greater susceptibility to proteolysis as acidity increased from p_H 5.5, whereas the isoelectric point of edestin is at p_H 5.5 to 6.0.

These varying results for the four substrates suggest much speculation on such lines as the state of molecular aggregation and osmotic pressure of the respective proteins at their isoelectric points and elsewhere, and the presence or otherwise of zwitterions or of un-ionised molecules in the various cases.

However, one observation is sufficiently striking to merit notice. It will be seen from the electrometric titration data given in Tables IV, VIII, XI and XIV that the p_H optimum for proteolysis of each protein approximates to that of maximum buffering power. The latter is given in the Tables, expressed as the Van Slyke coefficient $\Delta A/\Delta p_H$ or $\Delta B/\Delta p_H$, where ΔA and ΔB are increments of added acid and base, and Δp_H the corresponding change in p_H . The p_H range, corresponding to the maximum values of this coefficient for each protein, together with the results derived from the electrometric titrations of other observers, are summarised in Table XV. The only appreciable discrepancy is in the case of edestin, but even here the p_H optimum of proteolysis found is much nearer to that of buffering than to the isoelectric point. It seems that, when the p_H of maximum buffering power of the protein substrate falls within the range of activity of the malt proteinase, the optimum proteolysis will occur at or near that p_H . In the case of the proteins possessing an insoluble zone, the optimum is necessarily outside this insoluble zone. The proteinase appears to act on ionised protein, on both sides of the isoelectric point, and it is quite possibly specific for zwitterions where these exist. Further speculation is at present premature.

EXPERIMENTAL.

Preparation of the malt extract. Air-dried green malt was finely ground, extracted with 3 times its weight of water for 12 hours at laboratory tem-

perature and filtered. The extract used in the experiments, in which the formaldehyde titration was employed to measure proteolysis, was prepared from kiln-dried malt, which is unlikely to contain any quantity of the peptidases.

The substrates were crystalline egg-albumin (Merck), edestin from hempseed (Merck), caseinogen (a commercial product purified by Hammarsten's process), and fibrin prepared directly from blood and preserved in alcohol.

The buffer solution used in Exp. 2 was McIlvaine's phosphate-citrate, as described by Clark [1928].

All hydrogen ion measurements were performed by means of the quinhydrone electrode.

Exp. 1. Egg-albumin. Kinetics. 100 cc. of 6 % albumin + 20 cc. of acetate buffers (sodium acetate and acetic acid, $N/2$ with respect to each), p_H 4.6 + 50 cc. of malt extract, mixed and incubated at 46°. Control, the same with boiled malt extract. 25 cc. withdrawn at intervals and added slowly to 60 cc. of boiling water and boiled for 15 minutes, filtered, washed with boiling water, and total nitrogen determined in filtrate by the Kjeldahl process.

Table I.

Time (hours)	3	6	12	24	36
Action, cc. $N/10$ HCl (control deducted)	3.5	7.2	9.8	10.5	10.9

Exp. 2. Egg-albumin. Proteolysis at different reactions. 15 cc. of albumin + 15 cc. of malt extract + 10 cc. of phosphate-citrate buffer solution of appropriate p_H + $N/10$ HCl or NaOH as required + water to a total volume of 60 cc. After 8 hours at 46°, 30 cc. were withdrawn and coagulated as in Exp. 1, after addition of the required volume of $N/10$ NaOH or HCl. The latter was firstly calculated, and then a *pro rata* portion added to 20 cc. of the remaining solution and the resulting p_H tested to confirm that it was 4.8. The remaining portion of the reaction mixture was used for the determination of its p_H .

Table II.

p_H of reaction mixture	2.25	2.97	3.34	3.69	3.74	4.11	4.40	6.13	6.69	7.14	7.52	7.77
p_H of control	2.25	2.97	3.30	3.69	—	—	—	6.13	6.70	7.14	7.50	7.73
Proteolysis, cc. $N/10$ HCl (controls deducted)	1.0	5.5	8.6	8.0	7.9	7.0	6.4	1.2	0.8	0.6	0.5	0.3

Exp. 3. Egg-albumin. Proteolysis in absence of added buffers. This experiment was performed exactly as the previous one except that the phosphate-citrate buffers were omitted. All withdrawals were treated so as to contain the same concentration of NaCl at the time of coagulation.

Table III.

p_H of reaction mixture	3.87	4.32	4.73	5.28	6.18
p_H of control	3.73	4.30	4.73	5.28	6.19
Proteolysis, cc. $N/10$ HCl (controls deducted)	8.5	7.0	6.9	5.0	1.5

Exp. 4. Electrometric titration of the egg-albumin. Table IV gives the volumes of $N/10$ HCl or NaOH added to 1 g. of protein, and the p_H resulting. The buffer coefficients were calculated according to the formula of Van Slyke $\Delta A/\Delta p_H$ or $\Delta B/\Delta p_H$.

Table IV.

$N/10$ acid cc.	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0
p_H	4.89	4.55	4.30	4.06	3.87	3.67	3.49	3.31	3.09	2.64
$\Delta A/\Delta p_H$		1.47	2.00	2.08	2.63	2.50	2.78	2.78	2.27	2.22
$N/10$ base cc.	0.0	0.5	1.0	1.1	1.2	1.3	1.4			
p_H	4.89	5.31	6.03	6.27	6.55	6.83	7.09			
$\Delta B/\Delta p_H$		1.19	0.70	0.41	0.35	0.35	0.38			

Exp. 5. Caseinogen. Kinetics. 2 g. of caseinogen, suspended in 100 cc. of $N/20$ NaOH until dissolved, were brought to p_H 5.1 with $N/2$ HCl. 50 cc. of malt extract previously adjusted to p_H 5.1 were added, and the mixture incubated at 46° . At intervals 20 cc. were withdrawn, added to 20 cc. of acetate buffer solution (p_H 4.6), filtered, and total nitrogen determined on the filtrate. The control was treated similarly using boiled malt extract.

Table V.

Time (hours)	3.0	6.5	9.0
Action, cc. $N/10$ (control deducted)	4.2	7.8	10.7

Exp. 6. Caseinogen. Proteolysis at different reactions. 20 cc. of caseinogen (2 % in $N/20$ NaOH) + 15 cc. of malt extract + 19 cc. of $N/10$ HCl + respectively 0.0, 3.0, 5.0, 7.0, 8.0, 11.0 cc. of $N/10$ NaOH + water to 80 cc. Of this 20 cc. were withdrawn for p_H determination, and the remainder incubated at 46° for 8 hours. To each was now added the requisite volume of $N/10$ NaOH or HCl to bring to p_H 4.6 + water to the same total volume. Total nitrogen was determined on 50 cc. of the filtrate.

Table VI.

p_H of reaction mixture	2.58	2.97	3.35	3.82	4.10	5.34
Proteolysis, cc. $N/10$ HCl (controls deducted)	7.42	8.75	9.60	7.70	7.50	9.66

Exp. 7. Caseinogen. Proteolysis in presence of acetate. The procedure was exactly as in the previous experiment except that acetic acid was used instead of HCl.

Table VII.

p_H of reaction mixture	3.91	4.41	4.70	5.00	5.56	6.50	7.40
Proteolysis, cc. $N/10$ HCl (controls deducted)	7.6	6.2	6.6	7.9	11.7	5.16	2.2

Exp. 8. Caseinogen. Electrometric titration. Table VIII gives the volumes of $N/10$ HCl added to 0.2 g. of caseinogen in $N/10$ NaOH, and the resulting p_H . The buffer coefficients are also given.

Table VIII.

$N/10$ HCl cc.	8.0	8.4	8.6	8.8	9.0	9.2	9.4	9.6	9.8
p_H	8.37	6.89	6.47	6.31	5.93	5.49	5.15	4.71	4.16
$\Delta A/\Delta p_H$		0.27	0.48	1.25	0.53	0.45	0.58	0.45	0.36
$N/10$ HCl cc.		10.0	10.5	11.0	11.5				
p_H		3.80	3.36	3.00	2.60				
$\Delta A/\Delta p_H$		0.55	1.14	1.40	1.25				

Exp. 9. Edestin. Proteolysis at different reactions. 1 g. of edestin was suspended in 50 cc. of $N/10$ HCl until dissolved. 10 cc. of the solution + 15 cc. of malt extract + varying volumes of $N/10$ NaOH and water to total volume 40 cc. The mixtures were incubated at 37° for 14 hours. After withdrawing 10 cc. for the determination of p_H , the remaining 30 cc. were subjected to the formaldehyde titration. The controls were performed on malt extract + water, at the same values of p_H , and a control for edestin alone.

Table IX.

p_H of reaction mixture	4.0	4.4	4.6	4.9	5.1	5.5
Proteolysis, cc. $N/10$ NaOH (controls deducted)	2.65	2.80	2.75	2.70	2.55	1.50

Exp. 10. Edestin. Proteolysis in presence of acetate. The process was exactly as in Exp. 9 except that acetic acid was used instead of HCl. There was comparatively little protein in suspension in the reaction mixture, but a little was unavoidable in some cases.

Table X.

p_H of reaction mixture	4.25	4.40	4.64	4.82	5.05
Proteolysis, cc. $N/10$ NaOH (controls deducted)	1.65	1.40	1.35	1.25	1.20

Exp. 11. Edestin. Electrometric titration. Table XI gives the volumes of $N/10$ NaOH added to 0.05 g. of edestin, and the p_H resulting. The protein was dissolved in $N/10$ HCl.

Table XI.

$N/10$ NaOH cc.	4.0	4.2	4.4	4.5	4.6	4.7	4.8	4.9
p_H	2.41	3.37	3.54	4.04	4.47	4.82	6.58	8.58
$\Delta A/\Delta p_H$		0.21	1.18	0.20	0.23	0.28	0.06	0.05

Exp. 12. Fibrin. Kinetics. 2 g. of fibrin were dissolved in $N/5$ NaOH with heat, and brought back to p_H 6.8 and 4.6 in two portions. There was no precipitation at p_H 6.8, but precipitation took place at p_H 4.6. $N/2$ acetic acid was used for the purpose. Two experiments were performed, one on each solution, using 50 cc. of fibrin + 30 cc. of malt extract + water to 130 cc. Controls were treated similarly, using boiled malt extract. The reaction was checked by withdrawing 20 cc. at intervals, adding the required volume of $N/2$ acetic acid to bring to maximum precipitation and filtering. Total nitrogen was determined on the filtrates.

Table XII.

Time (hours)	1	2	4	
Action, cc. <i>N</i> /10 (controls deducted)	0.5	1.2	1.5	At p_H 4.5
	—	0.6	0.8	At p_H 6.9

Exp. 13. Fibrin. Proteolysis at different reactions. 5 g. of fibrin + 50 cc. of *N*/2 acetic acid were heated to just below boiling, allowed to stand for a time in the cold, filtered and the swollen fibrin dissolved in 300 cc. of *N*/8 NaOH by heating. By this means the fibrin was dispersed with less drastic treatment than in the previous experiment. It still precipitated, however, at p_H 4.8 on neutralisation, instead of p_H 6.8 which is the isoelectric point of fibrin. 20 cc. of fibrin + 15 cc. of malt extract + the required volumes of acetic acid or sodium hydroxide + water to total volume 80 cc. After 5 hours at 46°, 40 cc. were withdrawn and brought to p_H 4.8 by addition of the necessary volumes of acetic acid or NaOH, etc. as in *Exp. 12*.

Table XIII.

p_H of reaction mixture	3.81	4.20	4.58	5.00	6.03	7.59	8.27	9.00
Proteolysis, cc. <i>N</i> /10 HCl (controls deducted)	2.3	0.9	1.1	1.6	2.3	0.6	0.3	0.0

Exp. 14. Fibrin. Electrometric titration. The fibrin was dispersed as in *Exp. 12*. Table XIV gives the volume of *N*/10 HCl added to 0.1 g. of fibrin in *N*/5 NaOH. The buffer coefficients are also given.

Table XIV.

<i>N</i> /10 HCl cc.	9.0	9.3	9.5	9.6	9.8	9.9	10.0	10.2	10.4
p_H	7.60	6.22	5.91	5.46	4.86	4.16	3.80	3.11	2.89
$\Delta A/\Delta p_H$		0.22	0.64	0.22	0.33	0.14	0.28	0.29	0.91

Table XV. *Summary of p_H values corresponding to maximum values of buffer coefficients and proteinase activity.*

Protein	p_H range of maximum	
	Buffer coefficient	Activity of proteinase
Egg-albumin	3.31–3.67 (present communication)	3.3–3.6
	3.40–3.53 [Hendrix and Wilson, 1928]	
	3.10–3.50 [Loeb, 1921]	
Caseinogen	3.00–3.35	3.0–3.35 and 5.57
	and	
	6.31–6.47 (present communication)	
	3.10–3.41	
Edestin	and	4.25–4.4
	6.10–6.52 [Loeb, 1921]	
	3.37–3.54 (present communication)	
Fibrin	3.30–3.60 [Hitchcock, 1922]	3.81 or less and 6.0
	3.11–4.16 (present communication)	
	and 5.91–6.22	

SUMMARY.

1. The proteinase of green malt acts at 46° optimally on crystalline egg-albumin at p_H 3.3 to 3.6, on caseinogen at p_H 3.35 and at 5.57, on fibrin at p_H 3.8 (or less) and at 6.0, and on edestin at 37° optimally at p_H 4.3.
2. These values of p_H approximate in most cases very closely to those of the maximum buffering power of the protein measured at 18°.
3. The proteinase appears to act on ionised protein on both sides of the isoelectric point, but there is no clear evidence that it is specific for zwitterions, although this is quite possible in the cases of those proteins in which zwitterions exist.

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