CCXX. ENZYMIC ADAPTATION IN *CLOSTRIDIUM HISTOLYTICUM*

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RECENTLY Kocholaty & Hoogerheide [1938] described an example of the adaptation of certain of the endo-enzymes of the strict anaerobe *Cl. sporogenes*: by varying the *pH* of the culture medium a considerable shift in the *pH* optimum of some of the dehydrogenases could be produced. It has now been found that under specified conditions the *pH* optimum of the proteinase secreted by this micro-organism can be shifted, and that, by repeated subculturings on gelatin or casein, specific proteinases are developed which will hydrolyse gelatin, but not casein, and *vice versa*.

EXPERIMENTAL

The methods used in preparing the *Cl. histolyticum* proteinase and estimating its activity were the same as previously described [Kocholaty *et al.* 1938]. Casein (Hammarsten) and gelatin (Coignet Gold Label) were used as culture media, and as substrates for proteinase activity determinations. Bacteria-free Seitz filtrates of the incubated cultures were used in the enzyme studies.

Shift in the pH optimum of the proteinase

The proteinase secreted by *Cl. histolyticum* grown at *pH* 7.2–7.4 on the common media, such as neopeptone, horse meat broth, gelatin or casein, has a *pH* optimum of 7.0 as found by Weil & Kocholaty [1937]. The same holds for the proteinase secreted by various other types of bacteria. When *Cl. histolyticum* is grown on casein medium at *pH* 6.0 for 20 hr. the secreted proteinase is no longer optimally active at *pH* 7.0 but at about *pH* 6.7. During the 20 hr. incubation, the *pH* of the culture medium increases from 6.0 to 6.7 or 6.8. If the culture medium

Fig. 1. *pH* optimum of *Cl. histolyticum* proteinase. Abscissa: *pH*. Ordinate: proteinase activity in ml. of 0.1 *N* KOH. — = in acid medium (3% casein; 1% glucose). ---- = in neutral medium (3% casein). O = full activity. x = initial activity. Age of the culture: 20 hr.

is held at a definitely acid reaction during the whole 20 hr. of incubation a still larger shift in the *pH* optimum of the secreted proteinase takes place. A very simple method for achieving this effect is to add glucose to the culture medium. When *Cl. histolyticum* is grown on casein (3%) in the presence of glucose (1%),

extensive gas formation occurs as a result of sugar breakdown, and the pH of the culture medium drops, because of the formation of acids, from pH 7.4 to about 5.8 toward the end of the incubation period. The pH optimum of the proteinase found in filtrates from such cultures is now about 6.0. These facts are illustrated in Fig. 1. It will be seen that identical shifts occur in the pH optima both of the initial and the full (with cysteine plus Fe^{++}) proteinase activities.

This simple example of enzymic adaptability can be made still more striking. If a solution of 3% casein at pH 7.4 is inoculated with a culture of *Cl. histolyticum* grown on casein plus glucose as described above, the proteinase secreted after 20 hr. growth is again found to be optimally active at pH 7.0 (both initial and full activities). It is evident that the nature of the proteinase secreted by the bacteria adjusts itself very easily and rapidly to changes in the culture medium.

The production of specific proteinases by Cl. histolyticum

The proteinase of *Cl. histolyticum* readily attacks casein, gelatin, horse meat broth, egg albumin, neopeptone and clupein among the media investigated. The hydrolysis, in every case, is activated by SH-heavy metal complexes, for example, by cysteine plus Fe^{++} . When the bacteria are grown on casein or gelatin the secreted proteinase attacks both casein and gelatin to about the same extent (see Table I, Exp. 1).

Among the easily available proteins of high mol. wt., casein and gelatin show the greatest divergence in their amino-acid compositions. For this reason these two proteins were chosen for the following experiments: the greater the difference in the proteins studied, the easier it should be to establish a specific enzyme adaptation in the proteinase secreted by *Cl. histolyticum* when repeatedly sub-cultured on the respective proteins. The main differences in the amino-acid compositions of casein and gelatin are shown below, the values being those given by Hawk & Bergeim [1931].

Amino-acid	% in gelatin	% in casein
Glycine	25.5	0.4
<i>l</i> -Hydroxyproline	14.1	0.2
<i>d</i> -Valine	0.0	7.9
<i>d</i> -Hydroxyglutamic acid	0.0	10.5
<i>l</i> -Tyrosine	0.01	6.5
<i>l</i> -Tryptophan	0.0	2.2

By repeated transfers of *Cl. histolyticum* cultures from gelatin to gelatin, and from casein to casein medium, an attempt was made to develop specificity in the secreted proteinase for one or the other of these proteins. The transplantations were made as follows: inoculations of *Cl. histolyticum* from the stock culture were made in test tubes containing 10 ml. 10% gelatin or 10% casein. After bacterial growth had become well established,¹ 1 ml. portions of these incubation mixtures were transferred into fresh 10% gelatin and casein media, respectively. This operation was repeated 6 times, always transferring from gelatin to gelatin, and from casein to casein. The incubation period during these

¹ It was frequently found that *Cl. histolyticum* did not grow readily on gelatin medium. When ordinary gelatin was used an entire week often elapsed before bacterial growth began. This induction period could however be shortened by using a gelatin medium which had first been partially digested by trypsin, and then sterilized. Usually after one subculture on such trypsin-digested gelatin, the bacteria became sufficiently adapted so that satisfactory growth and enzyme secretion could be achieved on ordinary (not previously decomposed) gelatin. With casein, on the other hand, good growth and enzyme secretion were usually obtained after the first inoculation, no extensive induction period occurring.

sub-culturings varied from 24 to 48 hr., satisfactory bacterial growth occurring within this time. After the 6th transfer, larger amounts of 3% gelatin and 3% casein were inoculated with the respective cultures, and incubated for 24 hr. The solutions obtained after removal of the bacteria by Seitz-filtration were used in the enzyme studies.

Table I. *Formation of specific proteinases by Cl. histolyticum*

	Enzyme Substrate ...	G		G		C		C	
		Gelatin		Casein		Gelatin		Casein	
1	Initial activity	1.47		1.36		1.20		1.12	
	Full activity	2.51	*	2.69	*	2.34	**	2.43	**
2	Initial activity	1.18	1.12	0.38	0.54	0.46	0.70	1.00	0.97
	Full activity	2.16	2.00	1.88	1.81	1.80	1.79	2.27	2.24
3	Initial activity	1.28	1.20	0.00	0.40	0.00	0.37	1.18	1.12
	Full activity	2.50	2.68	1.20	1.28	1.47	2.00	2.32	2.41
4	Initial activity	0.30		0.18		0.24		0.41	
	Full activity	0.59		0.36		0.46		0.89	

Explanation of Table I:

G = Enzyme solution obtained by growing *Cl. histolyticum* on gelatin medium.

C = Enzyme solution obtained by growing *Cl. histolyticum* on casein medium.

Activity determinations were made on cell-free bacterial filtrates using gelatin and casein as substrates. Reaction mixtures contained 2 ml. filtrate, 3 ml. 6.6% casein or gelatin adjusted to pH 7.0, 5 ml. citrate-phosphate buffer (McIlvaine) at pH 7.0 and 1.6 ml. water (for initial activity) or 1 ml. neutralized cysteine-HCl (10 mg.) plus 0.6 ml. *N*/10 FeSO₄ (for full activity). Incubation time was 20 hr. at 40°. Estimations were made by the Van Slyke method, results being expressed in ml. 0.1 *N* base equiv. to the —NH₂ groups liberated.

Exp. 1. Comparison of proteinases obtained in casein and gelatin media after one inoculation. The bacterial filtrate was prepared from a culture of *Cl. histolyticum* grown for 24 hr. at pH 7.4 on 3% gelatin or casein.

Exp. 2. Specificity of proteinases obtained after 6 transplantations on 10% casein and 10% gelatin.

Column * = addition of 15 mg. *d*-valine, 13 mg. *l*-tyrosine, 4.3 mg. *l*-tryptophan and 21 mg. *d*-hydroxyglutamic acid to 10 ml. enzyme solution before activity determination.

Column ** = Addition of 50 mg. glycine and 28 mg. *l*-hydroxyproline to 10 ml. of enzyme solution before activity determination.

Exp. 3. Same as Exp. 2 except that 12 successive transplantations were made.

Exp. 4. Loss of specificity of *Cl. histolyticum* proteinases. Casein-trained bacteria (12 transplantations) were grown for 24 hr. on 100 ml. 3% gelatin supplemented with 150 mg. *d*-valine, 130 mg. *l*-tyrosine, 43 mg. *l*-tryptophan, and 210 mg. *d*-hydroxyglutamic acid. Gelatin-trained bacteria (12 transplantations) were grown for 24 hr. on 100 ml. 3% casein supplemented with 500 mg. glycine and 280 mg. *l*-hydroxyproline. After Seitz filtration, the enzyme solutions thus obtained (designated C and G, respectively) were tested for activity as described above.

As shown in Table I, Exp. 2, small differences are evident, after 6 transplantations, in the specificities toward gelatin and casein exhibited by enzymes developed respectively in gelatin and casein media. Particularly with reference to the initial activity, the gelatin-trained enzyme has lost some of its ability to hydrolyse casein, while the casein-trained enzyme attacks gelatin less readily than it does casein. By adding to the gelatin and casein enzymes, respectively, the more important amino-acids missing in casein and gelatin a slight increase in the initial proteinase activity towards these substrates is observed.

The transplantation of the above gelatin and casein cultures was continued as before, from gelatin to gelatin, and from casein to casein, until a total of 12 sub-culturings had been made. Then larger amounts of 3% gelatin and 3% casein media were inoculated, incubated for 24 hr., and the bacteria removed by Seitz filtration. Enzyme studies on the cell-free filtrates gave the results shown in Table I, Exp. 3. In the absence of added activator, the gelatin-trained enzyme

is no longer able to hydrolyse casein, and the casein-trained enzyme does not attack gelatin. Rather remarkable is the fact that addition of the activator, cysteine- Fe^{++} , reduces this specificity, so that the gelatin-enzyme now hydrolyses casein, and vice versa, the casein-enzyme attacks gelatin. Interesting also is the confirmation of the observation reported in Table I, Exp. 2, that the gelatin-enzyme can be somewhat activated towards casein by addition of the amino-acids missing from the latter, but present in gelatin. The same holds, *mutatis mutandis*, for the casein-trained enzyme.

When casein is inoculated with *Cl. histolyticum* which has been carried through 12 sub-culturings on gelatin, no bacterial growth takes place even in 2 weeks of careful incubation. The same holds true when gelatin is inoculated with the casein-trained bacteria. However, when the culture medium consists of gelatin plus those amino-acids which are missing from this protein but present in gelatin, the casein-trained bacteria will develop a fair growth in several days. Likewise the gelatin-trained bacteria will slowly grow on a casein medium, supplemented by the amino-acids absent from the latter but present in gelatin. As shown in Table I, Exp. 4, the enzymes secreted in these latter cases are able to attack both casein and gelatin again, although not very strongly.

DISCUSSION

For the sake of brevity, the specific enzyme obtained by repeated sub-culturings on gelatin is termed "gelatinase", and that obtained in casein medium, "caseinase".

The results just described are of considerable interest from several points of view. By systematically training *Cl. histolyticum* on media such as casein or gelatin it is possible to develop two distinct proteinases of such extraordinary specificity that they can sharply differentiate between casein and gelatin. Also by simply culturing the casein-trained bacteria on a gelatin medium supplemented by those amino-acids missing from gelatin but present in casein, an enzyme is produced which again attacks both proteins. The same result is achieved by growing the gelatin-trained bacteria on casein supplemented by amino-acids missing from this protein but present in gelatin. Such results indicate that the specificity of these proteinases must be of very labile quality, depending only on the nature of the proteins used in their production. Addition of simple amino-acids to the culture medium, in fact, suffices to cause the specificity differences to disappear. The results presented in Table I, Exp. 4, show how easily these "trained" bacteria, and their secreted proteinases, can revert to their original state.

Of particular interest is the behaviour of the specific proteinases toward cysteine plus Fe^{++} . In the absence of this activator, the "gelatinase" and the "caseinase" fail to attack casein and gelatin, respectively, but both substrates are hydrolysed by each of these enzymes when the activator is present. There are two possible explanations for this SH-Fe^{++} effect.

1. The initial activity of the proteinase undergoes an activation in the sense that an extension of the field of specificity of the enzyme occurs. In other words, SH-Fe^{++} -activation broadens the specificity range so that different types of proteins can be attacked.

2. A 2-enzyme system may be present, as once postulated for papain by Bergmann [1937]. In this case the initial activity would represent a proteinase I, which would not be influenced by SH-Fe^{++} . A second enzyme, proteinase II, would be inactive under normal conditions, and active only in the presence of

heavy metal-SH complexes, and could be perhaps a type of peptidase. Attempts are being made to decide between the two ideas. Preliminary results indicate that the first theory is probably correct.

Recently two reviews of previous work on the subject of enzymic adaptation in micro-organisms were presented by Yudkin [1938] and Karstroem [1938]. Both authors distinguish between "adaptive" and "constitutive" enzymes. Quastel [1937] holds the view that the so-called constitutive and adaptive enzymes do not represent entirely different classes of enzymes. They represent rather the limits of variability of enzymes in a cell, the constitutive having the least range, and the adaptive the greatest range, of variability under different environmental and nutritional conditions. Our own view goes still further: we are inclined to picture the differentiation between constitutive and adaptive enzymes as a function of time; the longer a micro-organism is trained under altered environmental or nutritional conditions, the more will the properties of the constitutive enzymes disappear, until finally complete adaptation occurs. The many examples of the dissociation of various bacterial strains indicate that this view is probably not far wrong.

Yudkin [1938] has developed an interesting "mass action theory" of enzyme formation in bacteria. He postulates the existence of various inactive enzyme-precursors in the bacterial cell, and explains enzymic adaptation not by the formation of new enzyme, but rather by the conversion of the already existing precursor into active enzyme. The addition of substrate results in shifting the equilibrium of the enzyme-precursor system in the direction of active enzyme formation. On this basis, the results presented in Fig. 1, on the shift in the pH optimum of the secreted proteinases, would have to be explained as follows: the cultivation of *Cl. histolyticum* on gelatin at a low pH results in the secretion of a proteinase, already present in the cell in the precursor form, which attacks gelatin optimally at an acid reaction; cultivation in neutral gelatin results in the secretion of a different enzyme, also present in inactive form, which splits gelatin optimally at neutral reaction. Such an assumption is difficult to justify. The only difference in the stimulations to which the bacterial cells are subjected in the two cases is the difference in the pH of the media; the substrate remains the same. A more reasonable theory appears to be that recently proposed by Quastel [1937] which treats the enzymes as "cell metabolites whose rates of formation and destruction follow the same physicochemical laws as those controlling the metabolism of any other metabolite of the cell".

The action of an enzyme depends essentially on its active group. On the basis of known facts, it may be tentatively suggested that perhaps not every enzyme present in the bacterial cell has its own characteristic colloidal carrier. It may be that only a limited number of different colloidal carriers are present, which are interchanged as necessity demands, and according to definite laws, among the active enzyme groups already present or formed during bacterial growth. The extent of substrate specificity can of course be influenced also by the more detailed structural characteristics of the colloidal carrier. The views of Quastel and Yudkin may perhaps be brought into harmony as follows: the "enzyme-precursor" postulated by Yudkin may be identical with the colloidal carrier which is in equilibrium with the various prosthetic groups; through various external influences these equilibria within the cell are disturbed, so that on the basis of the mass action law a coalition between colloidal carrier and active groups occurs, resulting in the formation of a new enzyme.

SUMMARY

Several examples of enzymic adaptation in the proteinase of *Cl. histolyticum* are described. By changing the pH of the culture medium it is possible to change the pH optimum of the secreted proteinase. By repeatedly subculturing the bacteria on gelatin or casein media, enzymes are finally obtained which hydrolyse casein but not gelatin and vice versa. The mechanism of bacterial enzyme formation is discussed.

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