ARTICLE:

THE AUTOLYSIS OF INVERTEBRATE TISSUES

Samuel Belfer, Pearl Koran, Howard Eder and H. C. Bradley

J. Biol. Chem. 1943, 147:345-354.

THE AUTOLYSIS OF INVERTEBRATE TISSUES

By SAMUEL BELFER, PEARL KORAN, HOWARD EDER, AND H. C. BRADLEY

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison, and the Marine Biological Laboratory, Woods Hole)

(Received for publication, November 10, 1942)

It has been well established that the gross mechanism involved in atrophy and autolysis is the same in all vertebrate tissues thus far examined (1). Proteolysis is accomplished by an enzyme complex called cathepsin. Further cleavage of the primary fragments is carried on by peptidases. While it is certain that there are large quantitative differences in the distribution of these enzymes in different tissues of the same species, and in the homologous tissues of different species, this field has not yet been adequately explored.

No systematic studies have been made to determine whether this or a similar mechanism is also to be found among the invertebrates, and very little is known concerning the phenomena of atrophy and tissue mobilization in this group. A number of isolated observations have been made, which indicate that an autolytic mechanism is present in invertebrate tissue. Thus Chen and Bradley (2) reported a comparative study of muscle autolysis which included squid and *Busycon* muscle, and indicated a low order of activity as compared with vertebrate muscle. Bishop (3) showed that the larval "fat body" of the honey-bee undergoes disintegration during pupation and showed an increased proteolytic rate at that time. Many studies have been made upon the *digestive* enzymes of invertebrates based upon their extraction from tissues. The interpretation of these results must remain doubtful, since the proteinases reported may be autolytic rather than food-digesting in function. In general also, these older observations were made before accurate control of the pH was possible and before the phenomenon of activation had been discovered. It will not be profitable therefore to review this literature. The more recent and accurately controlled experiments on invertebrate digestion have been reviewed recently by Vonk (4). In a number of investigations cathepsin is reported as a digestive enzyme, but its possible function in an autolytic rôle is not considered (5). The present study was planned therefore as a preliminary survey of the autolytic behavior of such invertebrate tissues as could readily be obtained in sufficient quantity at Madison, Wisconsin, and Woods Hole, Massachusetts.

Our sampling of the phyla is obviously limited. The two annelids examined were the common earthworm *Lumbricus terrestris* and *Nereis virens*,

representing respectively a land form and a sea form. Among the Mollusca we have studied the marine lamellibranchs *Venus mercenaria* and *Pecten magellicus* and the fresh-water forms *Lampsilis*, *Anadonta*, and *Lasmigona;* the marine gastropod *Busycon* was studied and the cephalopod *Loligo pealii*. *Homarus americanus* and the large edible shrimp *Peneus setifera* represent the Crustacea. *Limulus polyphemus* represents the Arachnids.

In the case of the worms and the lamellibranchs the whole organism was used. It is obvious that such digests represent the proteinases of the various tissues together with such digestive enzymes as may normally be secreted into the intestinal tract. The results obtained with *Nereis*, *Lumbricus*, and the lamellibranchs can only be provisionally interpreted, since it was not possible to separate the digestive system and its enzymes from the other tissues. In the other forms we were able to study single tissues, just as has been done with vertebrates.

EXPERIMENTAL

Digests were prepared and set up as described in a previous paper (1). Hemoglobin was added to some digests as a foreign protein of known fragility to supplement the tissue proteins themselves, of whose availability as substrate little is known. The presence of this extra substrate may serve to disclose a proteolytic enzyme even when the cell proteins themselves are not fragmented. Digests were maintained at the initial pH by frequent readjustments during the first 3 days of autolysis with the glass electrode. The pH levels used were 2.0, 3.0, 4.0, 5.0, 6.0, and 7.5. In the case of *Limulus* eggs intermediate levels were also used. Samples were precipitated by trichloroacetic acid of 5 per cent final concentration, and digestion measured by the increase in soluble nitrogen in the filtrates and by the increase of the tyrosine color reaction. In some digests we were unable to avoid the development of white precipitates with the tyrosine reagents, but, as they can be quickly removed by centrifugation and do not carry down the color compound, no inaccuracies of color measurement were involved. All digestions were carried on at 38° unless otherwise specified. In some cases the activating effect of cysteine and the inhibiting effect of KIO_3 were tried, to discover further similarity to vertebrate tissue proteinases.

Nereis—The whole organism was ground fine and homogenized. The digestion curve represents a composite of tissue proteinases plus those from the digestive tract and glands. From the shape of the curve (Fig. 1) it is clear that this mixed material contains proteinases predominantly of the tryptic type, with optimum pH at about 7 to 8. The fact that digestion goes on at pH 2 to 4, however, indicates the presence of proteinase

active in this more acid range. A very similar curve is given by hog pancreas under identical conditions, when both trypsin and cathepsin are known to be present (6). Cysteine and KIO_3 produced little activation or inhibition respectively. Raw hemoglobin is digested best at pH 8, which indicates that an enzyme is present not identical with mammalian trypsin, to which undenatured hemoglobin is resistant. For the present, the evidence indicates an active tryptase and a less active proteinase with an optimum in the region of pH 3 to 5.

Angleworm—The entire organism was used. Proteolytic activity (Fig. 2) is shown by this composite material between pH 3 and 7.5, with a definite optimum at pH 5. This also suggests several overlapping proteinases or a generalized enzyme active over a wide range.

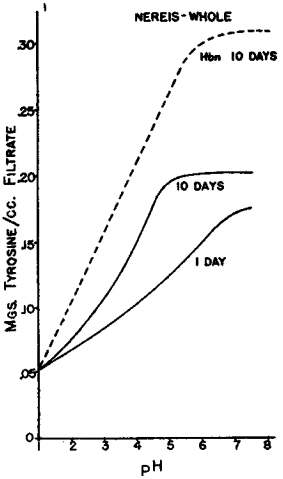

FIG. 1

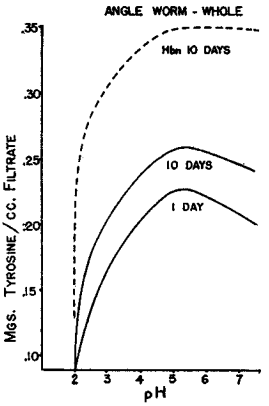

FIG. 2

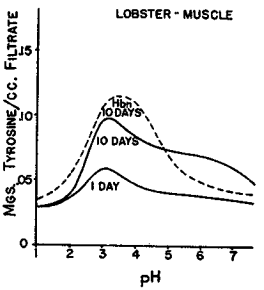

FIG. 3

Lobster Muscle—Material was obtained from the tail and leg muscles free from other tissues or secretions. Fig. 3 shows a low order of proteolytic activity with only a small digestion of hemoglobin in the 10 day period. A pH of 3 is optimum, with some digestion proceeding between pH 4 and 6. Cysteine did not activate digestion, though KIO_3 gave significant inhibition. This probably indicates complete activation of the system by the sulfhydryl compounds already present in the tissue. The failure of hemoglobin to be digested in the neighborhood of pH 6, while the muscle proteins are digested, seems to indicate a tryptase superficially not unlike mammalian trypsin. It is evident that lobster muscle protein can only be mobilized slowly for general use by this organism.

Lobster Digestive Gland—The hepato-pancreas of the lobster is believed

to be a true digestive organ secreting into the gastrointestinal tract. Our study of this tissue was preliminary and because of lack of time and material was adequate only to show that proteolysis at pH 4 is so rapid as to be nearly complete in 24 hours, with much greater liberation of tyrosine than in most of the invertebrate tissues we have examined. At pH 7.5 digestion is also quite good, though less rapid and complete. Cysteine shows a slight activation at pH 4 (Table I).

Shrimp Muscle—Refrigerated shrimps were obtained and the caudal muscle dissected free from the intestine and shell. The figures indicate a small total digestion, with a sharp maximum at pH 3 and no significant digestion at pH 5 and above, which is essentially the same as that for lobster muscle. Added hemoglobin was not digested at any pH tried.

Venus—Whole living clams were ground fine. The digestive mass, or midgut gland, makes up about 60 per cent of the entire soft tissues, and is richly supplied with the phagocytic cells which carry on the digestion of the

TABLE I

Lobster, Digestive Gland

	pH	Mg. tyrosine per 1 cc. filtrate				
		0 day	1 day	3 days	5 days	10 days
Control	7.5	0.09	0.27	0.29	0.30	0.33
"	4.0	0.09	0.40	0.43	0.49	0.49
" and Hb	4.0	0.09	0.60	0.63	0.63	0.63
" " cysteine	4.0	0.09	0.42	0.49	0.53	0.53

fine particles accepted as food by this form. According to Yonge (7) digestion of food proteins is accomplished by these wandering cells in the lamellibranchs and no proteolytic enzymes are secreted into the gut.

Two enzymes are clearly indicated (Fig. 4). The predominant one shows a maximum autolysis and digestion of hemoglobin at pH 3 to 4, with a great deal of activity even at pH 2+. This type of enzyme we have found characteristic of molluscan tissues. It resembles vertebrate cathepsin grossly and is strongly activated by cysteine and inhibited by KIO_3. A second maximum, much smaller, is found at pH 6.5, which suggests a tryptase.

Anadonta and Lampsilis—These medium sized, thin shelled, fresh-water clams are abundant in the Madison lakes and were obtained in the fall just before the winter resting period. The whole animals were used, and the curves of autolysis are very similar to those of the sea-clam just described. Digestion of the clam tissue is very rapid and is complete in 5 days. No tryptase was found.

Lasmigona, a thick shelled clam often weighing 400 gm. or more, gives an identical digestive pattern.

Pecten Muscle—The large adductor muscle of this bivalve is readily obtained refrigerated in the market. While not perfectly fresh, it had been kept frozen and probably represents the living muscle fairly well (8).

Fig. 5 indicates a very small amount of an enzyme similar to that of the bivalve just reported. Hemoglobin is digested slowly, which confirms the impression of a very small amount of enzyme present. Cysteine produces a slight activation and KIO_3 a slight inhibition. This tissue is strikingly stable. Although it makes up about half the total soft tissue of the animal, it evidently cannot be easily or rapidly mobilized for use by the organism as a whole. It is rich in glycogen, and acts as a fuel reservoir. Functionally it is rather inert as a contractile mechanism, though it is capable of making a series of rapid contractions by which the scallop occa-

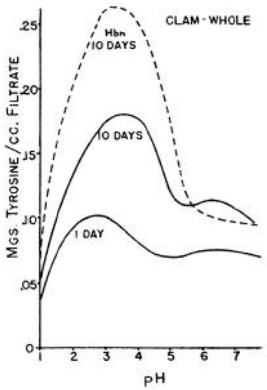

FIG. 4

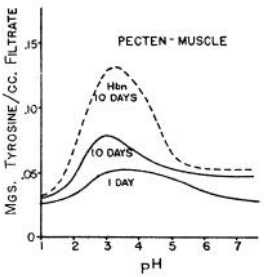

FIG. 5

sionally moves about. In this respect it is the most active bivalve muscle that we have studied.

Squid Tentacles—The squid is the most active of the invertebrates examined, and the most highly developed. It is in constant motion and capable of bursts of great speed for short periods. It cannot support a sustained high speed effort, however, and is quickly fatigued to the point of helplessness. It is said not to survive long in captivity because of its constant activity and the difficulty in providing food which it will take.

The tentacles were removed and immediately washed to remove salivary secretion ejected from the mouth in the premortal struggles. We suspect that the tryptic enzyme found in these digests was due nevertheless to such contamination.

Autolysis is much more rapid and extensive than in other molluscan muscles we have examined. In Fig. 6 the characteristic sharp peak is shown at pH 3.

Squid abdominal muscle shows almost exactly the same digestion curve

(Fig. 7) as that of the tentacles except for the fact that there is less evidence of a tryptase.

Busycon, Pedal Muscle—The pedal muscle of this large gastropod makes up nearly 50 per cent of the total animal, exclusive of the shell. When contracted it is a firm hard mass which can be easily obtained free from contaminating tissue or fluid. It is very difficultly dispersed, however, even by repeated grinding and homogenizing, and remains unsatisfactory for sampling. In the more acid digests the particles soften somewhat and become semigelatinous. There is little or no gross evidence of digestion under any conditions. Because of the difficulties of sampling, the individual results are subject to large errors. However, from many experiments, the curves shown in Fig. 8 are believed to be characteristic and reproducible.

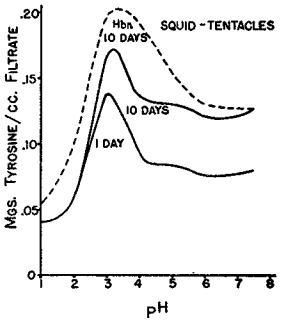

FIG. 6

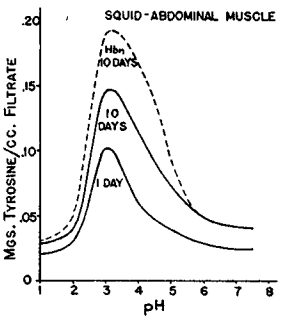

FIG. 7

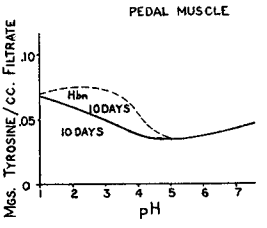

FIG. 8

There is very little autolysis in 10 days at any pH. There appears to be slight digestion in high acidity, which falls off to a minimum at pH 5 and shows a slight increase at pH 7.5. The slight increase of reactive tyrosine compounds is so small as to make it doubtful whether we are measuring autolysis. There does seem to be a slight digestion of hemoglobin between pH 1 and 3, which is probably evidence of a trace of active proteinase present. Altogether the pedal muscle of *Busycon* resembles most closely the inert adult connective tissues of the mammal, such as cartilage, yellow elastic and collagenous masses of ligaments, and tendons. Functionally the muscle is very slow moving and stands at the bottom of our muscle series so far as active contraction is concerned. It is well supplied with glycogen, like most molluscan muscle, but can hardly contribute protein significantly for general use of the organism. We have no data to indicate whether atrophic changes ever do occur in this muscle during starvation.

Albuminiferous Gland—A few ripe females were obtained in which the egg capsule-secreting gland was well developed and ready for capsule

formation. When ground, this tissue forms a stiff gummy mass rather hard to handle satisfactorily. It is loaded with the precursor of the protein that forms the insoluble string of "purses" within which the embryos develop. It was unlike any other glandular tissue we have examined, in that autolysis was very slight, with a maximum at about pH 3, of the same order as that found in the pedal muscle. There was considerable digestion of hemoglobin, however, at this pH, which indicates that the enzyme is not lacking but that the tissue proteins themselves are not available for cleavage by it (Table II).

The egg capsules themselves are composed of a highly insoluble protein, which is not digested in pepsin-HCl or active trypsin solutions. The gland presents, therefore, some interesting problems in protein transformation

TABLE II

Busycon, Capsule Gland

	pH	Mg. tyrosine per 1 cc. filtrate			
		0 day	3 days	5 days	10 days
Control	1.7	0.023	0.032	0.040	0.043
	3.0	0.023	0.055	0.057	0.058
	3.5	0.023	0.042	0.047	0.050
	4.5	0.023	0.026	0.026	0.037
	6.3	0.023	0.027	0.037	0.037
	7.5	0.023	0.031	0.042	0.041
" and Hb	1.6	0.025	0.058	0.064	0.072
	3.0	0.025	0.100		0.115
	3.3	0.025	0.100	0.135	0.155
	4.2	0.025	0.055	0.058	0.058
	7.5	0.025	0.033	0.045	0.054

from the viscous precursor to the insoluble final product of secretion, after it has come in contact with sea water.

Digestive Gland—The digestive gland of *Busycon* shows very strong proteolytic activity. With the exception of the hepato-pancreas of the lobster and the eggs of *Limulus*, it is the most active invertebrate tissue we have found. It shows the characteristic sharp peak at pH 3 common to all molluscan tissues we have examined, with a secondary low optimum around pH 6 to 7, indicating a tryptic enzyme present in small amount (Fig. 9). The gland itself has been reported to contain no trypsin-like enzyme (9), while the salivary glands do. It is believed to be absorptive rather than secretory in function. The low tryptic activity shown here is probably due to the presence of some salivary secretion in the absorbing diverticulae of the gland, which could not be entirely excluded from the material.

Limulus polyphemus—Several large females were secured heavily loaded with mature eggs. The latter could be separated easily from ducts and ovarian tissue, washed, and obtained uncontaminated. The muscle was dissected free from other tissues and from blood and hepato-pancreas.

Muscle—Muscle tissue autolyzes with a sharp optimum at pH 3. Digestion between pH 2 and 3 is rapid and nearly complete in 3 days. There is no digestion at pH 6 and 7.5, and only a very small proteolysis proceeding slowly at pH 5. Between pH 3 and 5, notably at pH 4, digestion proceeds slowly, but finally attains nearly maximum proteolysis (Fig. 10). Hemoglobin is digested best at pH 3, but not at all at pH 2 or 5. The sharpness of the digestive curve is the striking feature of this tissue.

Hepato-Pancreas of Limulus—The brown masses of this tissue can be readily freed from oviducts and eggs. It is homogenized to a smooth

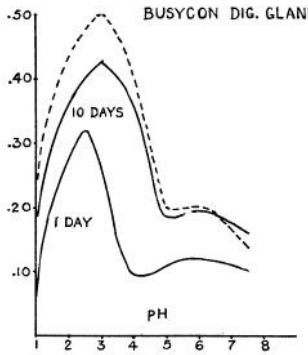

FIG. 9

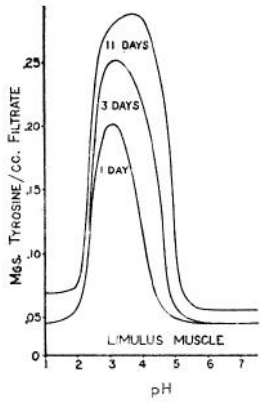

FIG. 10

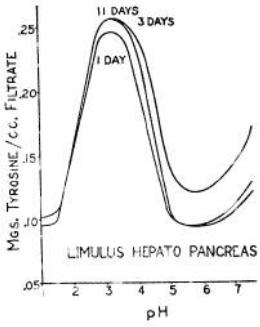

FIG. 11

suspension susceptible of accurate sampling. Two optima were found, one at about pH 3.5 which indicates a cathepsin, the other at pH 7.5 or more which indicates a tryptase (Fig. 11).

Eggs—The opaque gray-green eggs, with a diameter of about 2 mm., are obtainable in quantity from the gravid females during the early summer. By using screens it is possible to obtain the eggs entirely free from contaminating material. They are not easily ruptured even by fairly rough handling in the process of separation and cleansing. They are ground up easily into a smooth creamy suspension and are quickly homogenized to a uniform fine dispersion except for the tough egg membranes. The dispersions are so uniform and the particle sizes so small that digests may be sampled with the regular fine tipped pipettes.

A preliminary autolytic series showed that digestion was extraordinarily rapid and complete, and that activation by cysteine was considerable, as

was inhibition by KIO_3. A second series was set up with eggs obtained from two large females and maintained at a room temperature of about 24° in order to slow the digestion rate. After 12 days the digests were transferred to the warm room at 30°. The intermediate curves are therefore not strictly comparable to the others presented here, though the final digestion figures are.

As Fig. 12 shows, there is no digestion at all at pH 2. At pH 2.5 digestion is rapid. In the early hours of digestion the optimum is sharp and close to pH 3. It gradually shifts to pH 3.5 as the process continues, and when final equilibrium is approximated the optimum zone is broader and extends nearly to pH 4. Digestion is almost zero at pH 5, and there is no evidence

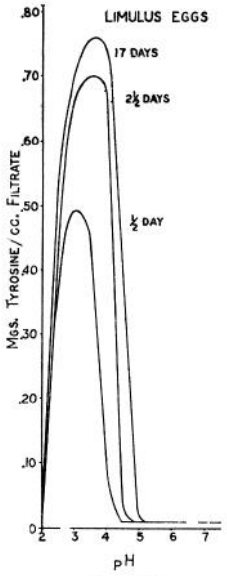

FIG. 12

of proteolysis beyond that point. It is evident that very small shifts in the H ion in the neighborhood of pH 4.5 will cause extraordinarily large differences in the amount of protein mobilized and in the speed of this mobilization. Cysteine increases the digestion rate and extent at all pH levels where digestion normally occurs. KIO_3 inhibits strongly, at the pH optimum, but digestion continues slowly. The results are strikingly similar to the digestion of mammalian liver for example, except that the curves are smoother, narrower, and more sharply defined. This is due in part to the fact that this material can be prepared in a homogeneous suspension which lends itself to accurate sampling and analysis. The symmetry of the curves and the narrow pH range suggest that a single protein consti-

tutes the substrate, or, if more than one protein is concerned, at least that isoelectric points and fragility of the proteins present are much alike.

The significance of the autolytic mechanism in the eggs appears to be clearly a provision for the mobilization of stored protein into amino acids for the synthesis of new tissue protein in the developing embryo.

DISCUSSION

From the results of the limited sampling of invertebrate tissues here presented, it appears probable that an autolytic mechanism exists throughout the invertebrate field, superficially at least like that found in all vertebrate tissues. Cleavage is initiated by a proteinase whose maximum activity is found to be close to pH 3, and whose effective range is from pH 2.5 to 5. In some tissue there may be additional proteinases which act best near the neutral point or in slight alkalinity. The amount of activity, or enzyme present, and the availability of the tissue proteins vary greatly. This variation finds its counterpart in mammalian tissues also. In comparing muscles of the several species of molluscs studied we find evidence that functional activity of the contractile mechanism correlates with its autolytic activity, as appears to be the case with vertebrate muscle.

As a general rule we have found invertebrate tissue proteinase to be susceptible of activation by cysteine and of inhibition by KIO_3. In some instances there is no activation, but the inhibitory effect of the oxidant is significant. In these cases it seems probable that the enzyme is already fully active when the tissue mince is prepared, and the brilliance of the nitroprusside reaction in such tissues is in harmony with this explanation.

Our tentative conclusion therefore is that a catheptic type of proteinase is widely distributed in invertebrate tissues and initiates the autolytic mobilization of the tissue proteins. Its optimum appears to be somewhat more acid than that found in vertebrates. In certain forms we believe the autolytic mechanism also functions as the digestive proteinase— specifically in the lamellibranchs in which a true proteolytic secretion into the gut is doubtful and in which the primitive device of phagocytosis appears to be the only provision for securing amino acids from the food particles.

BIBLIOGRAPHY

1. Bradley, H. C., *Physiol. Rev.*, **18,** 173 (1938). Bailey, B., Koran, P., and Bradley, H. C., *Biol. Bull.*, **83,** 129 (1942).
2. Chen, K. K., and Bradley, H. C., *J. Biol. Chem.*, **59,** 151 (1924).
3. Bishop, G. H., *J. Biol. Chem.*, **58,** 567 (1923–24).
4. Vonk, H. J., *Biol. Rev.*, **12,** 245 (1936–37).
5. Rosen, B., *Z. vergl. Physiol.*, **21,** 176 (1935).
6. Bradley, H. C., and Belfer, S., *Am. J. Digest. Dis. and Nutrition*, **3,** 220 (1936)
7. Yonge, C. M., *J. Marine Biol. Assn. United Kingdom*, **14,** 295 (1926–27).
8. Callow, E. H., *Biochem. J.*, **19,** 1 (1925).
9. Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, **13,** 17 (1905).