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University of New Hampshire, Ph.D., 1975
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STUDIES ON THE COMPOSITION AND AMYLOLYTIC

ACTIVITY OF THE MOLLUSCAN CRYSTALLINE STYLE

by

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M.Sc., University of Maine, 1969

A THESIS

Submitted to the University of New Hampshire
In Partial Fulfillment of
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Doctor of Philosophy

Graduate School

Department of Zoology

December, 1975

This thesis has been examined and approved.

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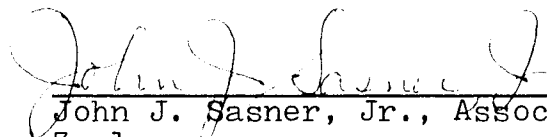
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I wish to thank the Biology Department of Bowdoin College, Brunswick, Maine, for permitting me to use their scanning electron microscope facility and particularly Dr. John Howland for his assistance and instruction in the use of the scanning electron microscope.

The contributions of my wife, Robin, were innumerable. Her kindness and understanding insured both the completion of this dissertation and the sanity of its author.

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ABSTRACT

STUDIES ON THE COMPOSITION AND AMYLOLYTIC
ACTIVITY OF THE MOLLUSCAN CRYSTALLINE STYLE

by

DAVID GIBSON TRAINER

The crystalline style is an acellular mucoprotein
rod found only in bivalve and herbivorous gastropod molluscs.
The enzymes contained within the style and the anatomical
association of the style with the digestive system indicate
that the style serves a digestive function. The present
study investigates several aspects of the composition and
amylolytic activity of the crystalline style of Mya arenaria.

    1. Histological techniques indicated that the iso-
electric point of the style is in the range of pH 4.0 to 4.5.

    2. The rapid dissolution of the style in alkaline
solutions and by proteolytic activity was demonstrated in
vitro. The possibility that the dissolution of the style
occurs through similar processis in vivo is discussed.

    3. Although citrate and EDTA are both strong calcium
chelators, style dissolution is increased by citrate and
decreased by EDTA.

    4. Antibodies formed against Mya arenaria whole
style protein precipitate some protein from style homogenates
of Placopectin magellanicus but none from Modiolus modiolus

style homogenates.

  5. Amylolytic activity rises with increasing chloride concentrations to a maximum activity at 0.5 M NaCl. The ability of other halides to activate style amylase is similar to their ability to activate mammalian amylase (Br > I > F).

  6. pH optima of style amylase are dependent upon the concentrations of chloride present and may occur at pH 6.3, 7.0, and 7.3.

  7. Amylase is partially inactivated after one hour at 25-35° C and totally inactivated after one hour at 45° C.

  8. Gel filtration of style extracts indicated that the major portion of style amylolytic activity is associated with low molecular weight species. Calculations based on gel filtration on Bio-Gel P-100 yielded approximate molecular weight values of 15,700 and 12,900 daltons.

  9. Electrophoresis of style extracts on agarose gels demonstrated three bands of amylolytic activity. Polyacrylamide disc electrophoresis demonstrated many more bands of amylolytic activity, but was not reproducible.

INTRODUCTION

Mya arenaria, an intertidal estuarine species,
utilizes a ciliary-mucous mode of feeding typical of other
lamellibranch bivalves. Particulate matter, drawn into the
mantle cavity through an incurrent siphon, is caught and
sorted primarily by the ciliary-mucous mechanisms of the
gills or ctenidia. Rejected particles are carried post-
eriorly by ciliary currents to be voided eventually through
the incurrent siphon. Acceptable particles are carried
anteriorly by separate ciliary tracts to the labial palps
which conduct further sorting before the accepted particles
are passed through the short esophagus to the stomach. The
stomach of M. arenaria, as other lamellibranchs, contains a
complex system of ciliary sorting areas and ciliary tracts
which may carry particles to sorting areas, to ducts leading
to the digestive diverticula, or to a midventral groove
leading to the midgut for elimination of specific particles
(Purchon, 1960). Digestion is carried out both extra-
cellularly in the stomach and by intracellular action in the
digestive glands.

The crystalline style is a non-cellular protein rod
found in the digestive systems of bivalve and herbivorous
gastropod molluscs. In M. arenaria one end of the crystal-
line style projects across the stomach and abuts against the
gastric shield, a chitinous structure on the left wall of
the stomach (Fig. 1). Halton and Owen (1968) and McQuiston
(1970) have shown that microvilli from underlying cells

1

Figure 1. Diagram showing position of the crystalline

style of <u>Mya</u> <u>arenaria</u>. G, gastric shield;

M, mouth; S, style; SS, style sac; ST,

stomach; VM, visceral mass. The alimentary

canal with the exception of stomach and

style sac is shown solid black. (From

Yonge, 1932.)
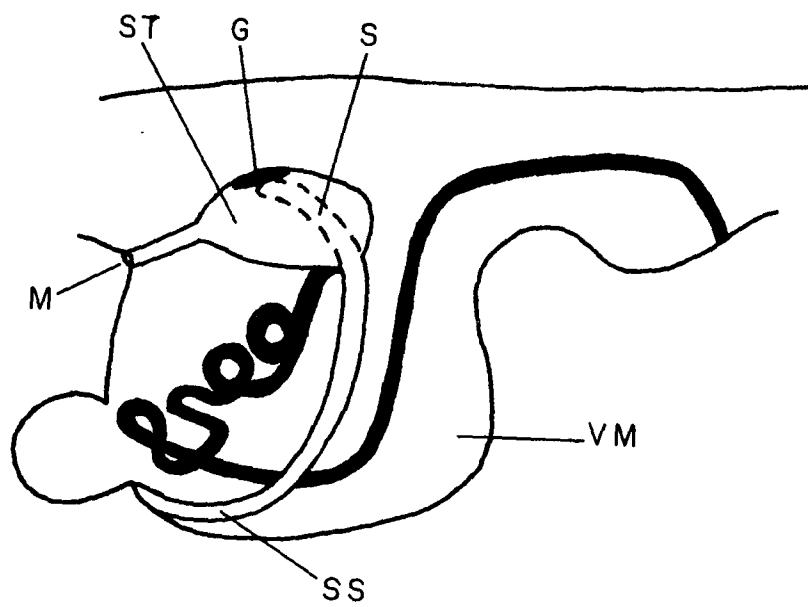
penetrate the shield and probably carry enzymes to the inner surface of the shield.

The style is both formed by and contained within the style sac, a pocket which may only open into the stomach at one end, as in M. arenaria, or which may also have a long-itudinal opening connecting it to the midgut. Rotation of the style by cilia lining the style sac has been observed and reported several times (Nelson, 1918; Yonge, 1926; Allen, 1958; Purchon, 1958). However, the failure of recent inves-tigators (Kristensen, 1972a; Bernard, 1973) to observe ro-tation of the style suggests that intermittent rotation occurs. The fact that styles are composed of concentric layers and the presence of a mucous thread wound around the tips of dissected styles indicate that the styles do rotate at some time. Rotation of the style may facilitate the di-gestive process through several mechanisms: 1. mucous material containing food particles may be "reeled" into the stomach from the esophagus, 2. mixing of the stomach contents by the style exposes more food particles to the stomach enzymes, 3. trituration of food particles between the style and gastric shield may occur. The most important feature of the style is probably the variety of enzymes contained within it (see review by Owen, 1966). However, the ability of the style to emulsify and decrease the viscosity of the stomach contents (Kristensen, 1972a) may also be important. Bernard (1973) states that the ability of the style to carry a thin layer of particulate matter into the style sac and press it against

the absorptive epithelial lining of the style sac represents
an important digestive mechanism.

The style is not homogeneous, but is composed of an
outer portion of concentric layers around an inner core of
longitudinal fibers (Fig. 2). Kristensen (1972a) has shown
that new style material is added to the outside of the style,
gradually moves into the central core, and is extruded from
the core into the stomach during the dissolution of the style
in vivo. The extruded anterior (stomach) end of the style
and the rounded posterior end are shown in Figures 3 and 4,
respectively.

Bailey and Worboys (1960) have shown that the organic
content of the style of Pinna nobilis consists of about 30%
carbohydrate and 70% protein material. Their amino acid
analyses revealed high proportions of proline and of hydroxy
and acidic amino acids. Carbohydrates found in styles of
several species by Bailey and Worboys (1960) or Doyle (1966)
include glucose, galactose, N-acetylated glucosamine and
galactosamine, fucose, xylose and deoxyribose.

Although styles of a few species may contain lipolytic
(George, 1952) or proteolytic enzymes (Reid, 1966) styles of
many species will hydrolyze a number of carbohydrate sub-
strates (Wojtowicz, 1972; Kristensen, 1972b; for review see
Owen, 1966). Amylase is apparently present in all styles
investigated and is probably of the alpha-amylase type
(Chaet, 1956; Owen, 1966).

Both mammalian (Frati and Caputo, 1970) and inverte-

Figure 2. Diagram of a transverse section through the
           style sac and intestine in a bivalve in
           which the style sac and intestine are in
           direct communication with each other.
           I, intestine; S, style; SS, style sac.
           (From Yonge, 1932.)

I

S

SS
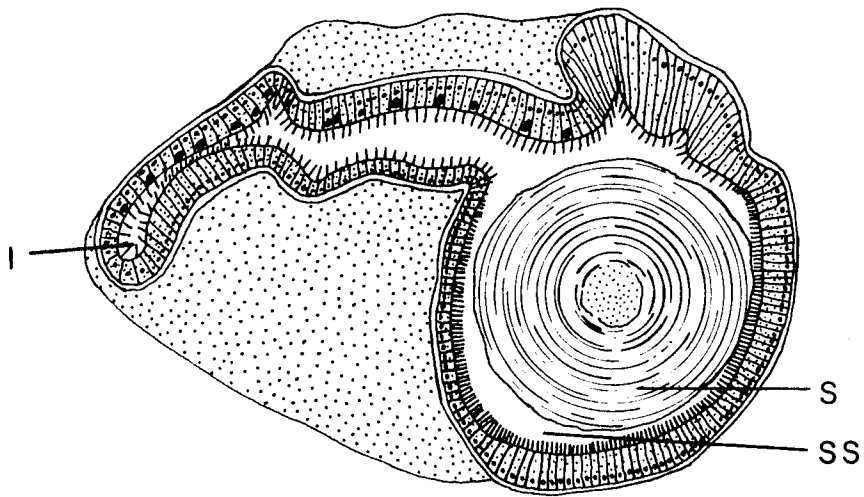
Figure 3. Scanning electron micrograph of the extruded
           anterior end of the crystalline style of <u>Mya</u>
           <u>arenaria</u> x 100.

Figure 4. Scanning electron micrograph of the posterior end of the crystalline style of _Mya arenaria_ x 200.

brate (Wojtowicz and Brockerhoff, 1972; Wojtowicz, 1972)
amylases have been isolated to an electrophoretically pure
state by successive Sephadex and Bio-Gel gel filtration
chromatography. Under certain conditions, the flow of
amylase through a Sephadex column is slowed by an apparent
affinity of amylase for the starch-like dextran matrix of
Sephadex. Because of its retarded flow, amylase is eluted
as though it were a smaller molecule than it actually is,
i.e. molecular weight determinations yield values lower than
the true molecular weight. Impurities eluted with amylase
thus have a lower molecular weight than amylase and can be
separated by gel filtration on a matrix with which amylase
will not interact, e.g. Bio-Gel.

The first part of the present study investigates
several aspects of the crystalline style relative to its
function in vivo. In the second part the characteristics
of the amylolytic activity of the style are studied and
compared to the characteristics of other invertebrate and
mammalian amylases.

STUDIES ON THE COMPOSITION OF THE CRYSTALLINE STYLE

INTRODUCTION

The crystalline style, a structure found only in bivalve and herbivorous gastropod molluscs, has been an object of continued interest since its description by Heide in 1686 (Yonge, 1932). The presence of various hydrolytic enzymes in styles has been reported by many authors (see review by Owen, 1966) and is indicative of its digestive nature. The style is housed within a style sac and composed of material released from typhlosoles within the style sac (Kato and Kubomura, 1954; Goreau et al., 1973). Styles of some species are of a mucous-like consistency and may rapidly dissolve in vivo or in vitro. In other species, styles are more solid and stable. Styles of the first type have been related to species in which the style sac has a longitudinal open connection with the midgut. In species with a persistent style, as Mya arenaria, the style sac is open only at one end where the style protrudes into the stomach (Yonge, 1923). Doyle (1966) and Bailey and Worboys (1960) have studied the biochemical nature of the crystalline style. The outer part of the style is composed of concentric layers or lamellae, while the central core portion appears as longitudinally oriented fibers. Kristensen (1972a) has shown that during dissolution of the style in vivo the core portion is extruded from the anterior (stomach) end of the style and is thus dissolved

independently from the outer lamellar portions. In this communication we report additional observations on the physical nature of the style of _Mya_ _arenaria_ and discuss the possible relationships of these features to the style's function _in_ _vivo_.

MATERIALS AND METHODS

Amylase activity was determined by the method of Bernfeld (1955), employing a substrate of 1% soluble starch (Fisher) and 0.5 M NaCl in 0.1 M sodium phosphate buffer pH 7.0. The procedure of Lowry _et_ _al_. (1951) was used for protein determination.

For the production of antibodies, homogenates of whole styles from _M_. _arenaria_ were injected subcutaneously into a rabbit six times at intervals of one week. Each injection contained approximately 450 µg of style protein and was administered as a mixture of equal volumes of style homogenate and Freund's adjuvant.

Immunoelectrophoresis was performed according to techniques described by Ouchterlony (1968). Gels, layered on 3 x 11 cm glass plates, contained 1% Difco Bacto Agar in 0.025 M barbital buffer, pH 8.6. Electrode vessels contained 0.05 M barbital buffer pH 8.6 and were electrically connected to the gels with filter paper strips. After 20 µl samples of whole style homogenates were added to the surface of the gel, electrophoresis was carried out for approximately 2 hours at

3 mamp per plate. Proteins in the gels were stained with 0.05% Coomassie Blue after precipitation with 12% trichloro-acetic acid, or were exposed to the antiserum prepared above by filling a center well with the antiserum.

RESULTS

Isoelectric Point

The approximate isoelectric point of the style was determined by a histochemical technique (Ruthmann, 1970). Separate solutions of orange G and methylene blue stains were prepared in individual 0.02 M acetate buffer solutions ranging from pH 3.5 to 8.0 in increments of 0.5 pH units. Single styles were incubated in each of the buffer-stain solutions for 10 minutes. The failure of the styles to stain with orange G or with methylene blue at pH 4.0 and 4.5 in-dicates an isoelectric point in this pH range.

Solubility of the style as a function of pH

Individual styles were placed in 1.5 ml of 0.01 M acetate or phosphate buffer at various pH values and incubated at room temperature for 16 hours. After removing the whole styles or centrifuging to remove small undissolved style particles for later assays of residual material, 0.5 ml aliquots of the buffer solutions were assayed for amylase and soluble protein. Aliquots for amylase determinations were first brought to pH 7.0 by addition of 0.5 ml of 0.1 M sodium phosphate at pH 7.0.

The residual style material, not dissolved during the 16 hour incubation above, was solubilized by incubation at pH 8.0 for 24 hours and assayed for protein and amylase as above. The results shown in Figure 5 demonstrate the greater amounts of protein and amylase dissolved and lower amounts of residual protein and amylase remaining after incubation at higher pH values.

Enzymatic degradation of the style

Individual styles were placed in separate enzyme solutions, each containing 5 mg of commercial enzyme (Sigma Chemical Co.) dissolved in 5 ml of either 0.1 M phosphate buffer, pH 7.0, or 0.02 M acetate buffer, pH 5.0. The degree of dissolution of each style was visually estimated and recorded at various times during incubation at room temperature. The results recorded in Table 1 demonstrate a rapid dissolution of the style by proteases and little or no degradation of styles incubated with carbohydrases.

Calcium and its role in style dissolution

Because of the role of calcium in structural protein, the presence of calcium and the effect of its removal were investigated. Flame emission spectrophotometry of two acid hydrolyzed styles yielded values of 0.408 and 0.403 µg Ca/mg whole style. Using the data of Doyle (1966) for M. arenaria styles (0.16 mg dry style/mg whole style, 0.5 mg protein/mg dry style), the above values were converted to 5.1 and 5.0 µg Ca/mg protein.

Figure 5. Amylase and protein dissolved and not dis-
solved from crystalline styles incubated at
room temperature for 16 hours in buffers at
the indicated pH values.

●————————● amylase dissolved

●— —— —— —● amylase not dissolved

○————————○ protein dissolved

○—— —— — —○ protein not dissolved
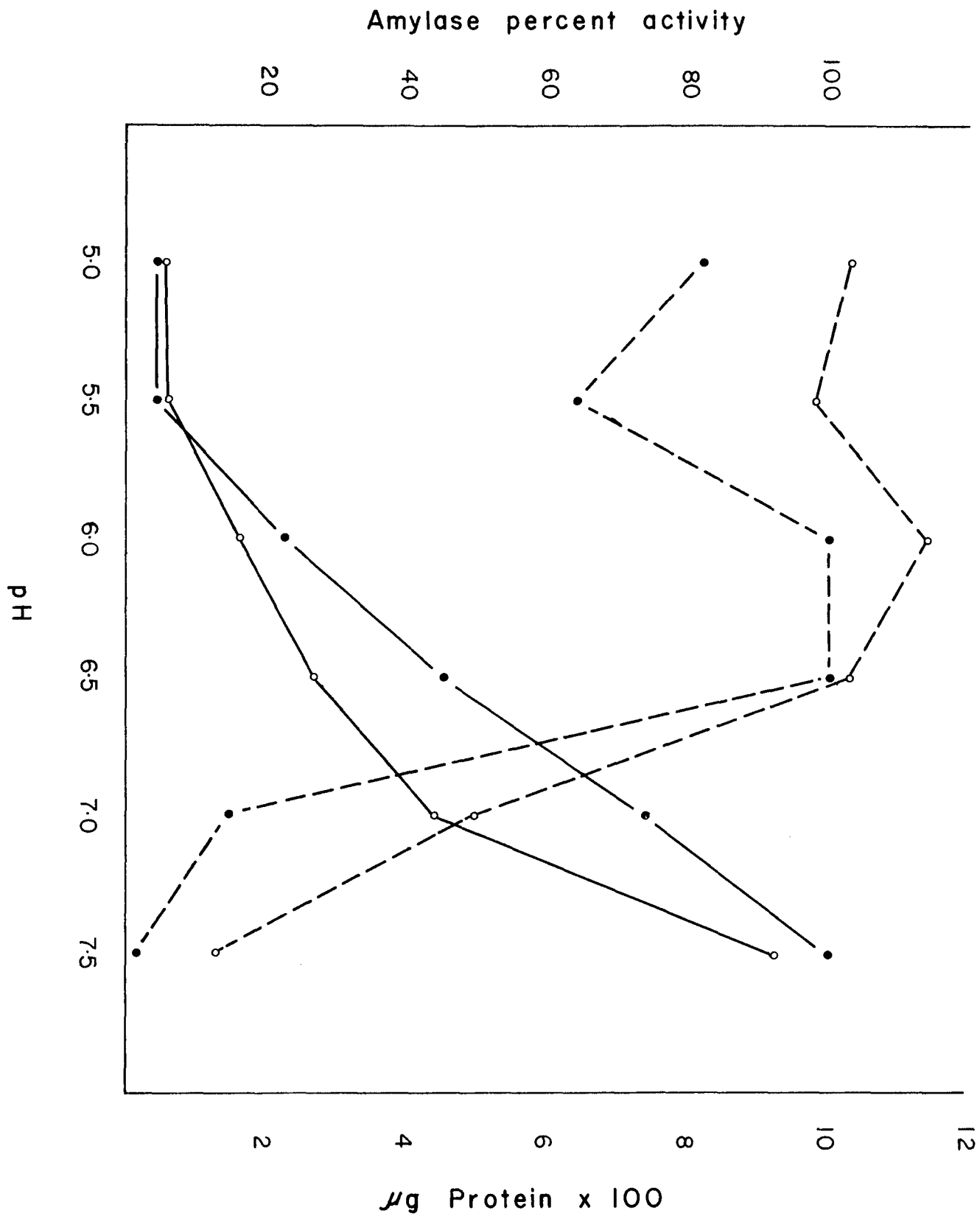
Table 1. Dissolution of crystalline styles by various enzymes.

Enzyme	pH	Time		
		0.5	1	7 Hr.
Buffer only	7.0	+++/+++	+++/+++	+++/++
Hyaluronidase	7.0	+++/+++	+++/+++	++/++
Lysozyme	7.0	+++/+++	+++/+++	++/++
Pancreatin	7.0	+/+	0/0	0/0
Pronase	7.0	+/+	0/0	0/0
Subtilisin	7.0	+/+	0/0	0/0
Trypsin	7.0	0/0	0/0	0/0
Alpha glucosidase	7.0	+++	++	
Beta glucosidase	5.0	+++/+++	+++/+++	+++/++
Beta galactosidase	7.0	+++/+++	+++/+++	+++/++
Buffer only	5.0	+++/+++	+++/+++	+++/+++

Style 1/Style 2; +++ = intact style: 0 = completely dissolved.

Dissolution of styles in the presence of calcium
chelators, citrate and ethylenediamine tetraacetate (EDTA)
was measured and compared to dissolution in phosphate buffer.
Styles were placed in separate solutions of: a. 0.01 M phos-
phate buffer, pH 6.0; b. 0.01 M phosphate buffer, pH 6.0,
containing 10^{-2} M EDTA; and c. 0.01 M citrate buffer, pH 6.0.
After incubation for 18 hours at room temperature, the amount
of protein dissolved in citrate buffer was 242% of that
dissolved in phosphate buffer, indicating a more rapid dis-
solution of style material in citrate. However, styles
incubated with EDTA had a stiffer, more solid consistency
than any of the others. And, the style protein dissolved in
the presence of EDTA was only 44% of that dissolved in phos-
phate buffer without EDTA.

Immunoelectrophoresis

The style proteins of Mya arenaria were compared to
those of the horse mussel, Modiolus modiolus, and the scallop,
Placopecten magellanicus, by immunoelectrophoresis as de-
scribed in the materials and methods section using antisera
against M. arenaria whole style homogenates. Staining for
protein with Coomassie Blue after electrophoresis in a Difco
Bacto Agar medium demonstrated that some M. arenaria style
protein migrated toward the anode electrophoretically, while
others were carried toward the cathode by endosmosis (Fig. 6).
Nearly all of the P. magellanicus style proteins migrated
toward the cathode (Fig. 6). Antiserum prepared against
whole style protein of M. arenaria contained antibodies

Figure 6. Proteins of styles of <u>Mya</u> <u>arenaria</u> (left) and <u>Placopecten</u> <u>magellanicus</u> (right) stained with Coomassie Blue after electrophoresis on a Bacto-Agar immunoelectrophoresis plate. Anode is toward top of photograph in Figures 6 through 9.
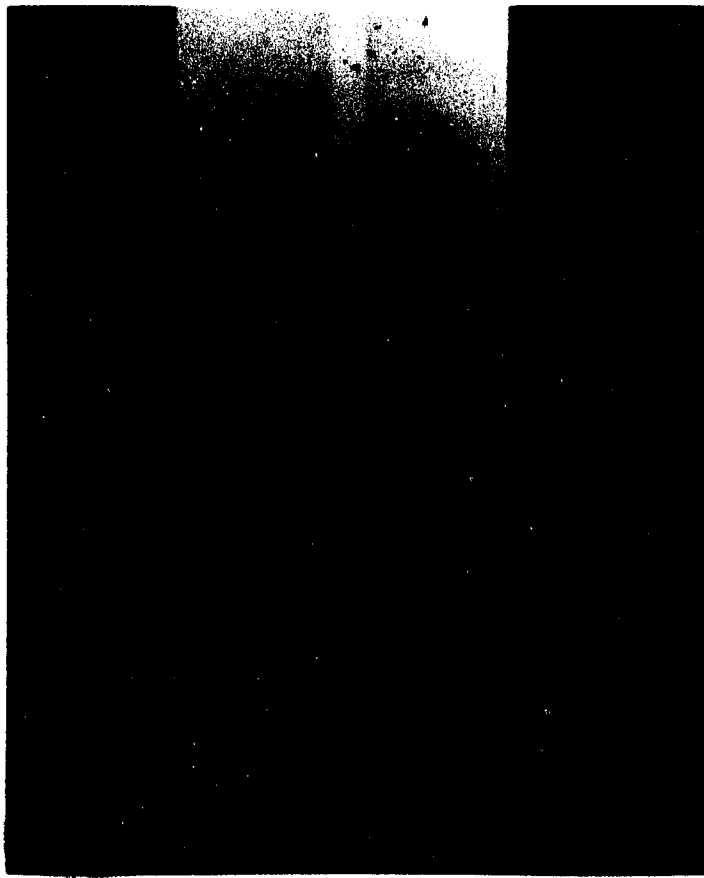
which precipitated several specific proteins of the M. arenaria style but which precipitated only one protein of the P. magellanicus style (Fig. 7). Proteins of M. modiolus migrated toward the cathode (Fig. 8), but showed no cross reaction with the antibodies formed against M. arenaria style proteins (Fig. 9).

DISCUSSION

The approximate isoelectric point of pH 4.0-4.5 determined here is in agreement with the observations of Bailey and Worboys (1960) that the minimum solubility of the crystalline style of Pinna nobilis occurs at about pH 5.

The rapid dissolution in vitro of M. arenaria styles with increasing pH (Fig. 5) supports observations of the dissolution of styles from other species (Morton, 1969; Kristensen, 1972a). Although several reports (Yonge, 1925, 1926; Morton, 1971) indicate that the stomach pH of various bivalves is acidic (pH 5.4-5.8) and invariant, Morton (1969) found that the stomach pH of Dreissena polymorpha varies from 6.6 to 8.2. Thus a rise in pH of the stomach contents, presumably resulting from an influx of sea water, may serve as a mechanism for dissolution of the style in vivo.

The rapid degradation of styles by proteases (Table 1) suggests another mechanism of style degradation in vivo. Proteases have been identified in the stomach contents of several bivalves including Mya arenaria (Reid, 1966, 1968). Kristensen (1972a) has shown that extracts of the bivalve

Figure 7. Immunoelectrophoretic pattern of style
proteins of <u>Mya</u> <u>arenaria</u> (left) and
<u>Placopecten</u> <u>magellanicus</u> (right) after
precipitation with antiserum against
<u>M</u>. <u>arenaria</u> whole style protein.
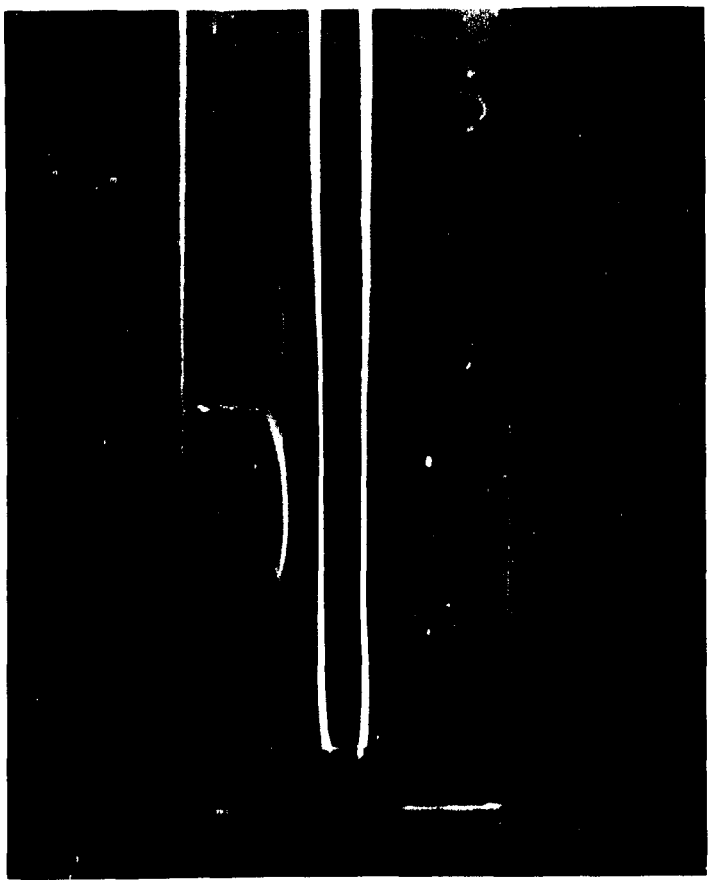
Figure 8. Proteins of the styles of _Mya_ _arenaria_
(left) and _Modiolus_ _modiolus_ (right)
stained with Coomassie Blue after electro-
phoresis on a Bacto-Agar immunoelectro-
phoresis plate.
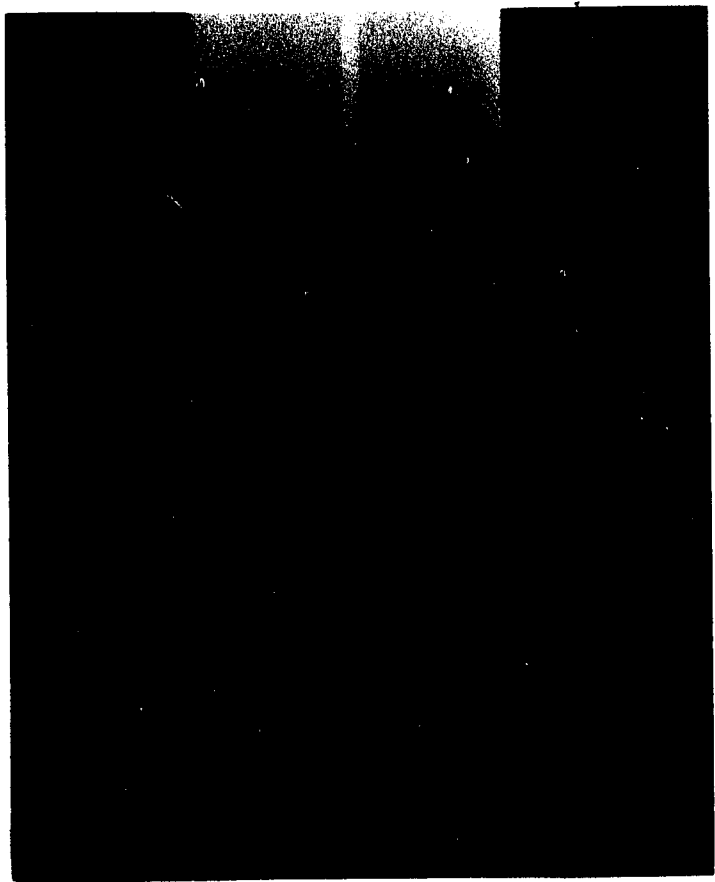
Figure 9. Immunoelectrophoretic pattern of style
proteins of Mya arenaria (left) and
Modiolus modiolus (right) after precip-
itation with antiserum against M. arenaria
whole style protein.
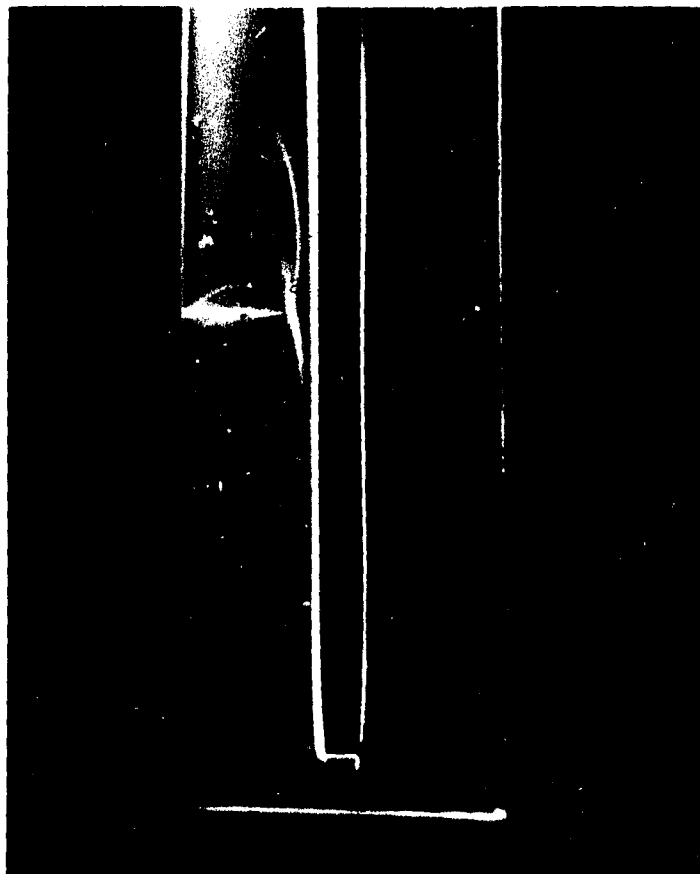
digestive diverticula increase the rate of style dissolution in vitro. The digestive diverticula contain proteases as well as other enzymes (Reid, 1966, 1968) which may be active in the stomach after their release from excretory spherules derived from the digestive diverticula (Morton, 1971).

Halton and Owen (1968) demonstrated esterase, acid phosphatase, and amino peptidase activity in the gastric cuticle, a homologue of the gastric shield, in the proto-branchiate bivalve Nucula sulcata. Although McQuiston (1970) did not demonstrate enzymatic activity he did determine that the fine structure of the gastric shield of Lasea rubra is similar to that of the protobranch's gastric cuticle. Because the style is dissolved in vivo in the general area contacting the shield (Kristensen, 1972a) the possibility of style degradation by gastric shield enzymes should be studied further.

Citrate and EDTA are known to chelate calcium and, to some degree, other divalent cations. Thus the opposite effects of citrate and EDTA on the dissolution rate of styles in vitro are difficult to understand.

The styles of different molluscan species are known to vary with respect to their consistency and their stability, or solubility, in sea water. While the style of Mya arenaria is quite stiff and relatively stable, the style of the scallop Placopecten magellanicus is softer and more soluble. The style of the horse mussel Modiolus modiolus is softer and less persistent than that of P. magellanicus. Because these

characteristics of styles probably result in part from their component proteins, the proteins of _M. arenaria_ styles were compared to those of the other two species. The results demonstrate that at least one component of the style of _M. arenaria_ has a very similar counterpart in the style of _P. magellanicus_. Whether the immunological similarity of styles is generally correlated with similarities of physical characteristics remains to be seen.

AMYLOLYTIC ACTIVITY OF THE CRYSTALLINE STYLE
OF MYA ARENARIA (BIVALVIA, MOLLUSCA)

INTRODUCTION

The molluscan crystalline style contains enzymes which are able to hydrolyze a number of different carbohydrate substrates (Wojtowicz, 1972; Kristensen, 1972b; for review see Owen, 1966). Lipolytic activity has been demonstrated in the style of Crassostrea virginica (George, 1952). Endopeptidase isozymes have been reported in the style of Cardium edule (Reid, 1966). An amylolytic activity in style material was demonstrated as early as 1900 (Coupin) and has since then been shown to be one of the most active enzymes found in the style. Animal amylases are generally believed to be α-amylases (Owen, 1966), and those of the crystalline style are more similar to α-amylases than β-amylases in terms of molecular weight (Wojtowicz, 1972), pH optima (Black and Pengelley, 1964; Horiuchi and Lane, 1966; Kristensen, 1972b; Wojtowicz, 1972), and migration in paper chromatography (Chaet, 1956). Wojtowicz (1972) has purified and characterized amylase from the style of the scallop Placopecten magellanicus. The purpose of the present study was to investigate certain characteristics of the amylolytic activity in the style of Mya arenaria and to compare these characteristics to those of amylases found in other invertebrate or mammalian systems.

MATERIALS AND METHODS

Clams were purchased from a local distributor and maintained without feeding in recirculating water tanks at 12° C. After removal, styles were homogenized in phosphate buffer or 0.85% saline or were prepared as an acetone powder. Acetone powder was prepared according to Bundy and Gustafson (1973) but using larger volumes of organic solvents than was prescribed. Whole homogenates and resuspended acetone powders were centrifuged at 5,000 g for 5 minutes to remove undissolved style material. Dialysis, when employed, was carried out for a minimum of 24 hours against four changes of distilled water. Lyophilized powder was prepared from resuspended acetone powder after centrifugation and dialysis (Wojtowicz, 1972).

Amylase activity was determined by the method of Bernfeld (1955), employing a substrate of 1% soluble starch (Fischer) and 0.5 M NaCl in 0.1 M sodium phosphate buffer pH 7.0 unless otherwise noted. The procedure of Lowry et al. (1951) was used for protein determination.

Descending gel filtration was performed in 1.5 x 90 cm columns. Eluents of 0.1 M sodium phosphate buffer, pH 7.0, or 0.85% NaCl were used with Sephadex G-100 at a flow rate of 6-8 ml/hr; with Bio-Gel P-100, 0.1 M sodium phosphate buffer, pH 7.0 was eluted at 2-4 ml/hr. Columns and fractions were maintained at 2-4° C. Molecular weights were determined as described by Andrews (1964).

Disc electrophoresis on Canalco (Rockville, Md.)

apparatus with standard RDS reagents was performed using 7%

acrylamide gels run at pH 9.5 for approximately one hour at

5 mamp per gel. In a second electrophoretic method, 1%

agarose in 0.05 M Barbitol buffer pH 8.6 was layered on

3 x 11 cm glass plates and allowed to solidify. Samples

of 20 μl were applied to the surface of the gel. Electro-

phoresis was run for 3 hours at 4 mamp per gel using barbitol

buffer as above. Proteins were stained with Coomassie Blue

(0.05%) after precipitation with 12% trichloroacetic acid.

In order to locate amylolytic activity in disc electrophoresis

gels, an acrylamide separating gel containing 0.25% starch

(Bedford and Reid, 1969) was employed. After electrophoresis

as above, the gels were incubated at room temperature for one

hour in 0.1 M sodium phosphate buffer pH 7.0 with 0.5 M NaCl.

Gels were then placed in a dilute iodine-potassium iodide

solution in which the unhydrolyzed starch stained blue and the

area hydrolyzed by amylase remained clear. Amylase activity

was identified in agarose electrophoresis gels by infiltrating

the gel with starch after electrophoresis and then incubating

and staining as above.

RESULTS

Halide Activation

Amylase activity of a centrifuged dialyzed 1% style

homogenate was increased by addition of NaCl to a maximum

activity at 0.5 M NaCl in sodium phosphate buffer, pH 7.0

(Fig. 10). Amylase activity decreased with higher concen-

Figure 10. Amylase activity in the presence of
varying concentrations of sodium chloride.
Maximum activity (at 0.5 M NaCl) is con-
sidered 100% activity. Amylase activity
of dialyzed homogenate (16.5%) is indicated
in the lower left of the figure.
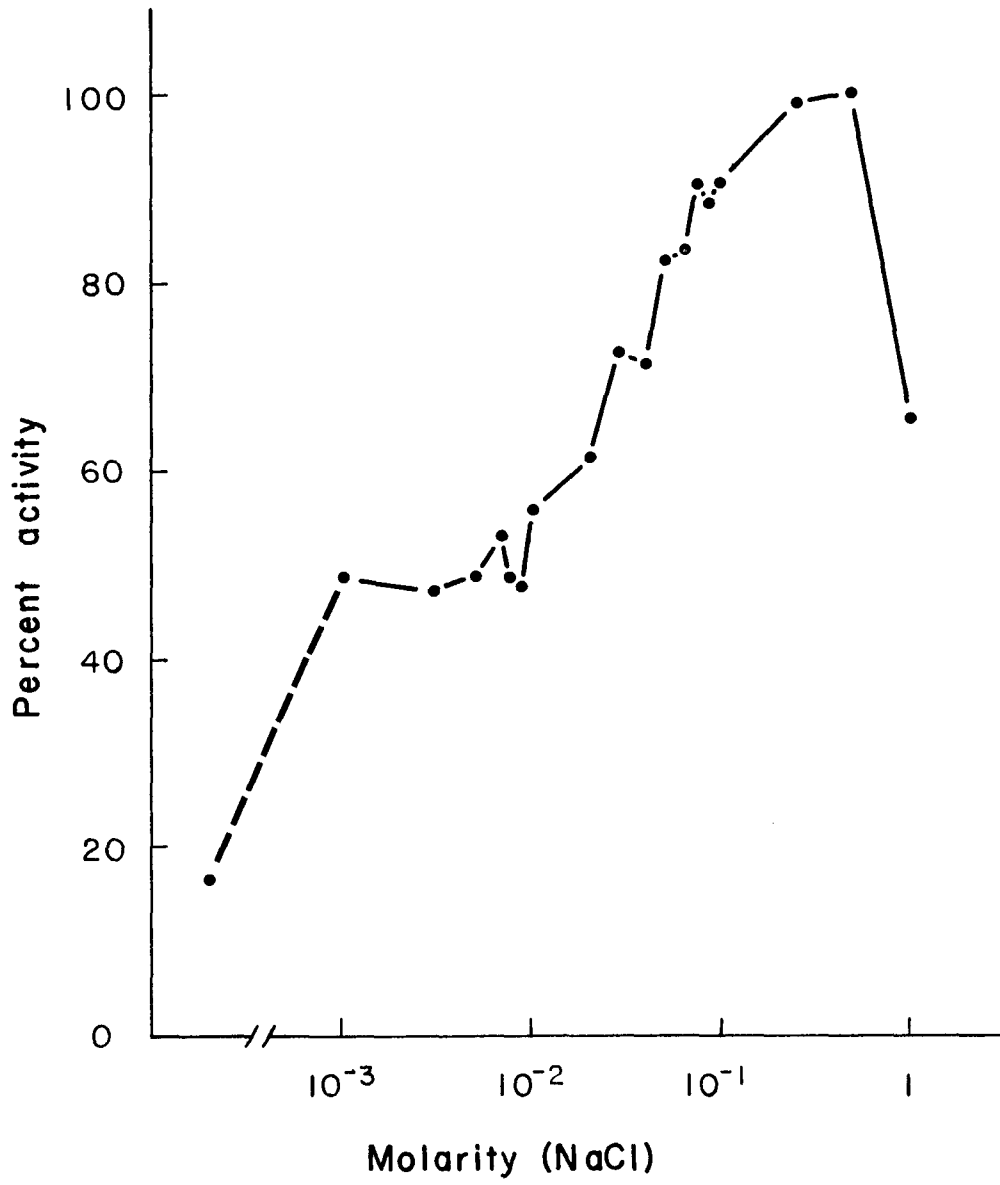
trations of NaCl. The plateau seen in Figure 10 between 10^{-3} and 10^{-2} molar NaCl was reproduced in several experiments.

In assays using substrate buffered with Tris-HCl instead of sodium phosphate the amylase activity of dialysate incubated with 0.1 M NaCl was 93% of that incubated with 0.1 M NH_4Cl indicating that the increased amylase activity seen in Figure 10 results from chloride and not sodium ions.

Experiments similar to those above demonstrated the effect of other halides on amylase activity in the absence of chloride (Table 2). Bromide increases the amylase activity of a dialized homogenate almost as much as chloride, iodide less, while fluoride may have a slight inhibitory effect.

pH Optima

Frozen styles were homogenized, centrifuged and dialyzed. Figure 11 illustrates the amylase activity of the homogenate at varying pH and in three concentrations of NaCl (0.003 M, 0.1 M, 0.5 M). In the presence of 0.5 M NaCl, the highest activity was found at pH 7.0. Peaks of activity occurred at pH 6.3 and 7.3 in 0.003 M and 0.1 M NaCl. The peaks at pH 7.3 (0.003 M and 0.1 M NaCl) decreased markedly in each of three successive determinations.

Heat Stability

Aliquots of style homogenate were placed in ice or water baths at various temperatures from 0° C to 100° C for one hour, then assayed at 25° C for amylase activity.

Table 2.   Substitution of other halides for chloride.

Salt	Concentration	Amylase Activity (% of activity with NaCl)
NaBr	0.1 M	95
NaBr	0.05 M	93
NaI	0.1 M	65
NaI	0.05 M	52
NaF	0.1 M	16
NaF	0.05 M	20
none (dialysate)	--	19

Figure 11.   pH optima of style amylase at three
             concentrations of NaCl.
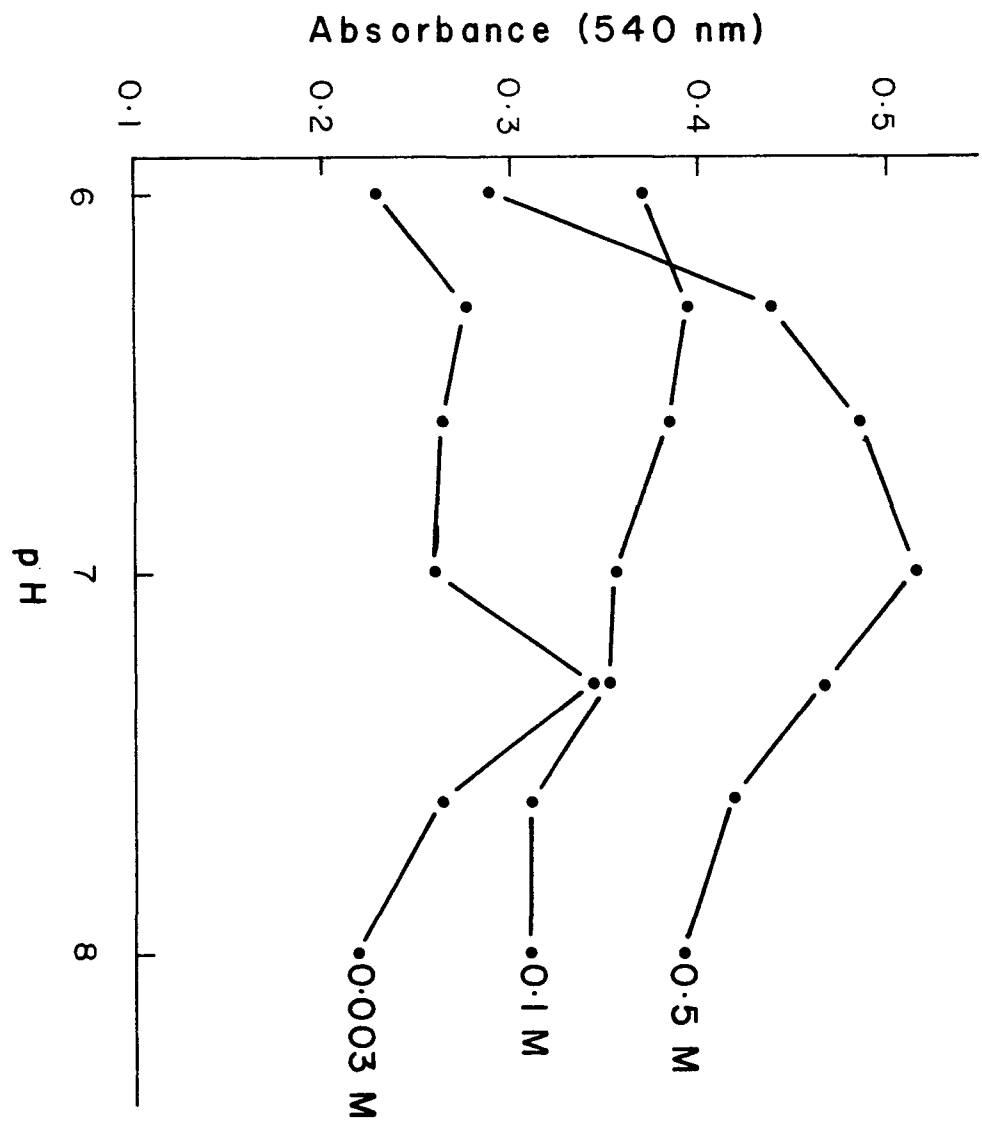
Samples held at 0° C and 15° C had equally high activity
while those held at 45° C or higher showed no amylase
activity (Fig. 12).

Gel Filtration

Gel filtration of various style extracts on Sephadex
G-100 and Bio-Gel P-100 produced elution patterns which were
similar with respect to the elution of protein or of amylo-
lytic activity (Fig. 13). A broad peak of protein was always
eluted at the void volume. Two peaks of amylolytic activity
were found. Peak A, a broad peak containing a very low
activity, was eluted at or near the void volume. The second
peak, B, had a much higher amylolytic activity and had a
high elution ratio (Ve/Vo = 2.0 - 2.9).

When lyophilized style extract (100 mg in 1.0 ml)
was filtered on Sephadex G-100 utilizing a 0.1 M sodium phos-
phate pH 8.0 buffer, the elution ratio (Ve/Vo) of peak B was
2.0. Under similar conditions, the use of a 0.85% NaCl
eluant, rather than the sodium phosphate buffer above,
apparently increased the affinity of amylase for the dextran
matrix of Sephadex and thus retarded the flow of amylase
through the column. The B peak eluted as two close peaks,
B1 and B2, with elution ratios of 2.60 and 2.93. Molecular
weights corresponding to these elution ratios were below the
range calibrated with known proteins but extrapolated values
were below 10,000.

Because amylase does not interact with the acrylamide
matrix of Bio-Gel, molecular weight determination using this

Figure 12. Heat stability of amylase. Style homo-
genates were held at the temperature
indicated, then assayed for amylase
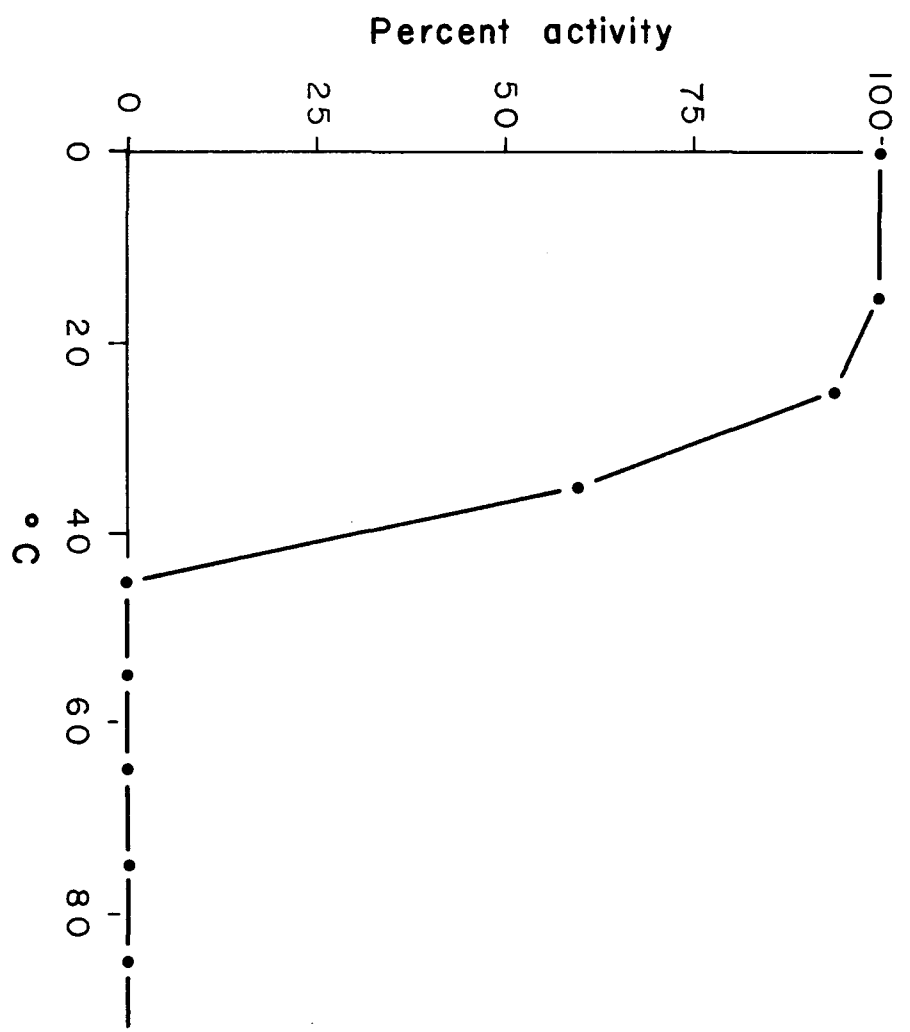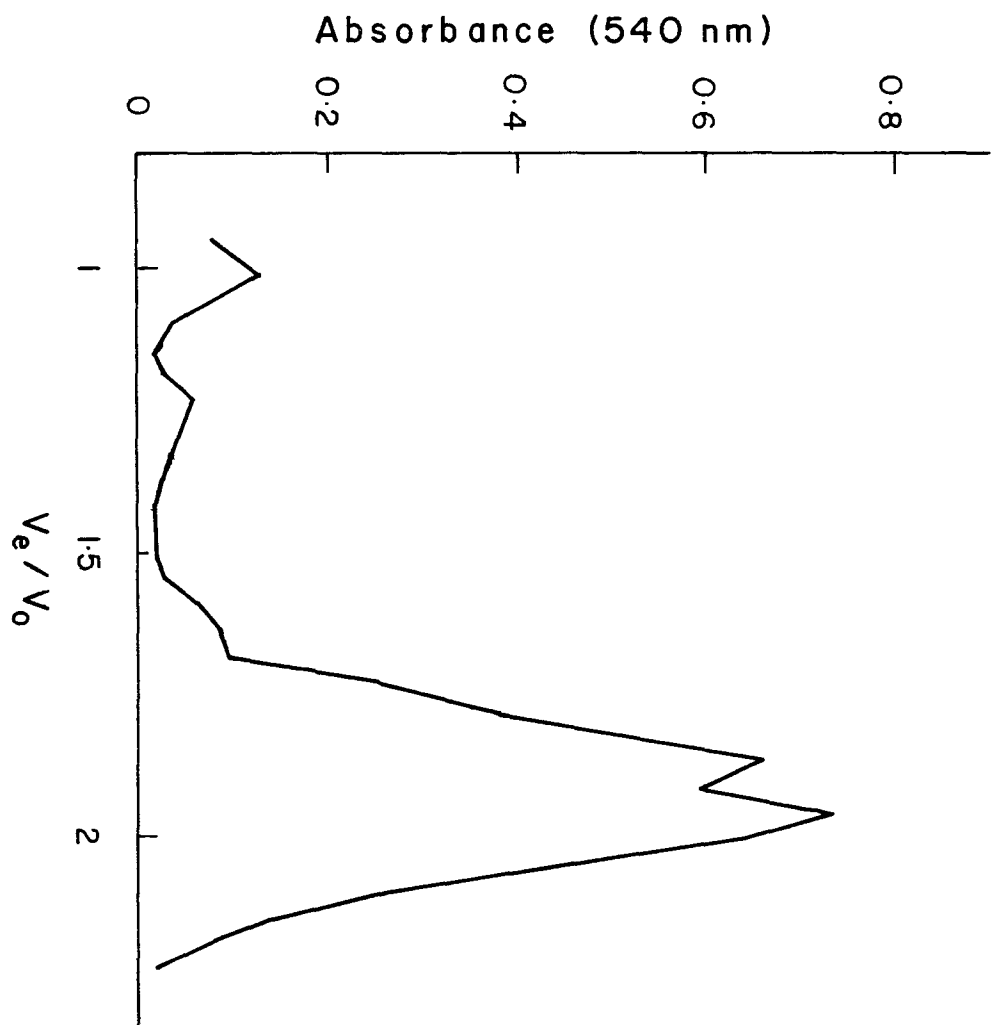activity at 25° C.

Percent activity

Figure 13. Gel filtration of amylolytic activity.
The results shown are from the gel fil-
tration of whole style homogenate on a
Bio-Gel P-100 column, employing a pH 7.0
sodium phosphate buffer, 0.1 M.

matrix should yield more accurate values. Approximate molecular weight values for peaks B1 and B2, calculated on Bio-Gel P-100 by comparing elution ratios of the amylolytic peaks to those of known proteins, are 15,700 and 12,900 respectively (Fig. 14).

Electrophoresis

Polyacrylamide disc electrophoresis of whole style extract demonstrated a minimum of ten protein bands. Electrophoresis of peak B eluted from a Sephadex column with sodium phosphate buffer yielded six protein bands. When the B peaks eluted from two Sephadex column runs were concentrated and passed through a Bio-Gel column, disc electrophoresis of the resulting Bio-Gel B peak produced two protein bands. Two protein bands were also seen after electrophoresis of the B peak from gel filtration of whole style extract on Bio-Gel. However, the patterns of two protein bands from the two samples above were similar with respect to only one band.

Disc electrophoresis on starch-impregnated gels produced as many as six bands of amylolytic activity from samples of whole style extract or concentrates of those gel filtration fractions eluted at the void volume (peak A). Similar electrophoresis of peak B concentrates produced two or three bands of amylolytic activity. Although the results obtained were not adequate to indicate the number of amylase species present nor the ways in which they might associate, the results do serve to demonstrate the complex nature of the amylolytic activity in the style.

Figure 14.   Molecular weight determination of amylase

by comparison of the elution ratios of

amylase shown in Figure 13 to those of

proteins of known molecular weight.   BSA

represents bovine serum albumin.
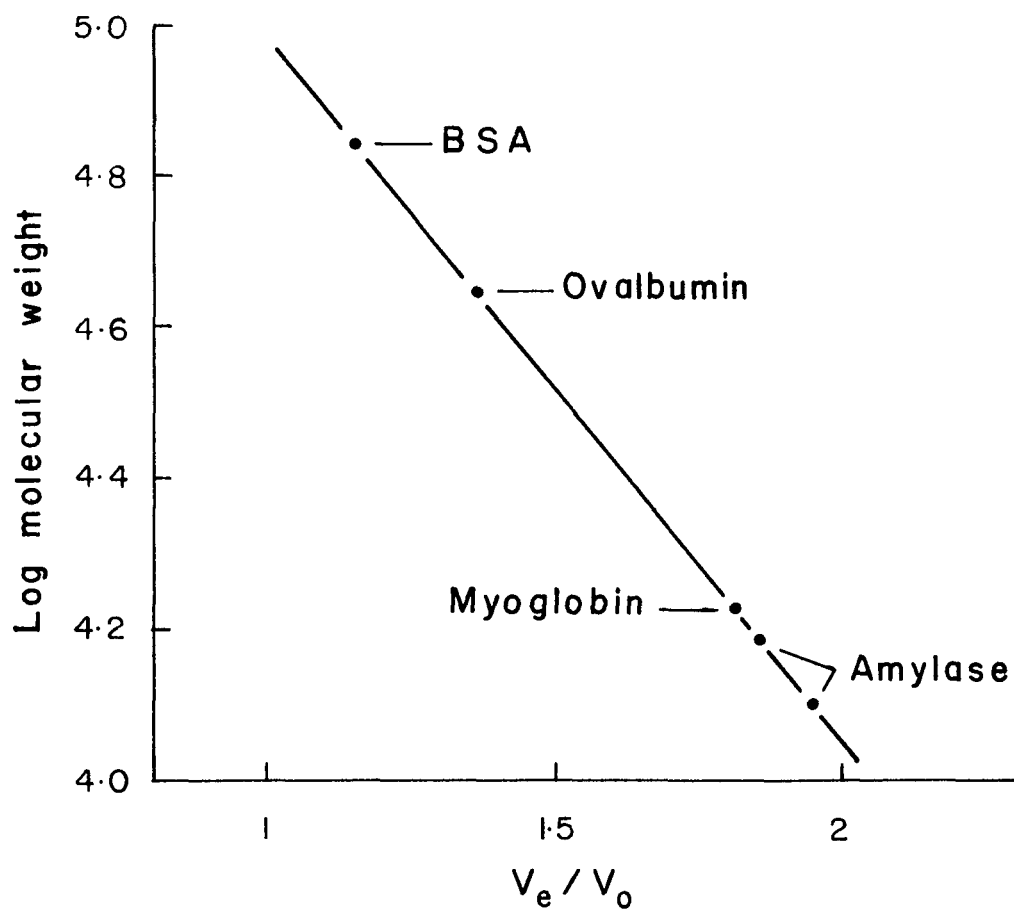
Electrophoresis of whole style protein on agarose plates produced one large rapidly moving protein band, a second narrower band midway between the first band and the origin, and a continuous smudge between the origin and the rapidly migrating band (Fig. 15). Electrophoresis of whole style extracts or gel filtration peak A concentrates on agarose consistently demonstrated three bands of amylolytic activity (Fig. 16). The migration of one of these bands corresponded to that of the large rapidly moving protein band. Only one band, that closest to the origin, was visible after electrophoresis of peak B.

DISCUSSION

The style amylase of M. arenaria appears particularly well suited to the NaCl concentrations at which it must function. The optimum NaCl concentration shown in Figure 10 (0.5 M) is nearly equal to that contained in "normal" 35‰ salinity sea water. An estuarine species such as M. arenaria can be exposed to much lower salinities, and Figure 10 indicates that some amylase activity is retained at very low NaCl concentrations. Because M. arenaria is an osmoconformer, as are most marine molluscs (see review by Robertson, 1964), the NaCl concentration of the medium in which the amylase acts will equal that of the surrounding water.

The activity plateau between 10^{-3} M and 10^{-2} M NaCl (Fig. 10) is indicative of more than one chloride binding site, i. e. one site is able to bind chloride at low NaCl

Figure 15. Electrophoretic pattern of whole style proteins after electrophoresis on a 1% agarose gel and staining with Coomassie Blue.
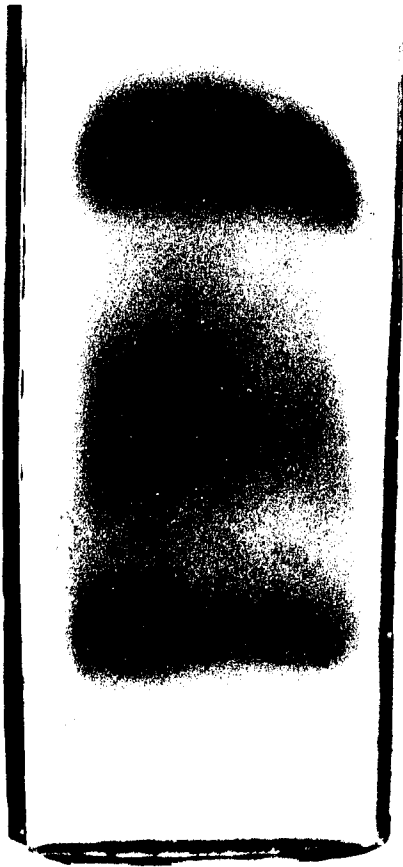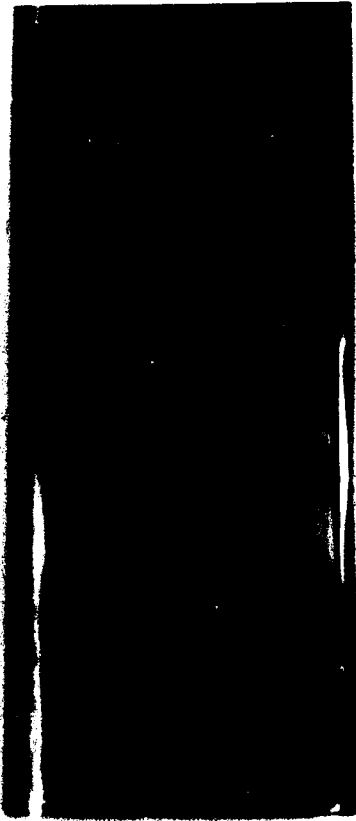
Figure 16. Electrophoretic pattern of amylase from
            whole style homogenate after electro-
            phoresis on a 1% agarose gel. Light
            bands indicate the areas of amylolytic
            activity.

concentrations (10^{-3} - 10^{-2} M), while the binding at a
second site increases as the NaCl concentration increases
from 10^{-2} M to 0.5 M.   Because of the electrophoretic demon-
stration of different amylase molecules (Fig. 16), I believe
that the different chloride binding sites occupy different
amylase molecules rather than multiple sites on a single
amylase molecule.

The activation of mammalian amylase by other halides
in the absence of chloride (Br > I > F) (Schwartz and Fleischer,
1970) is very similar to that found in the present study
(Table 1).

The pH optima of style amylase shown in Figure 11
(6.3, 7.0, 7.3) is in the range found in other molluscs (6.2
and 7.0, Black and Pengelley, 1964; 5.8-5.9 and 6.5-6.7,
Horiuchi and Lane, 1966; 6.5, Wojtowicz, 1972) and in mammals
(6.5-7.5, Schwartz and Fleischer, 1970).   Although different
pH optima at different chloride concentrations may be inter-
preted as resulting from the effect of the chloride on the
tertiary structure of one amylase enzyme, the activation of
different amylase molecules of different pH optima seems
more likely.   Indeed, three amylases, with pH optima of 6.2,
6.6, and 6.9, were separated by Berk et al. (1963) by ion
exchange chromatography of rabbit serum.   The presence of a
highly labile amylase with a pH optimum of 7.3 may have
resulted in the decreased activity observed at pH 7.3 in
successive experiments.

Both mammalian (Frati and Caputo, 1970) and inverte-

brate (Wojtowicz and Brockerhoff, 1972; Wojtowicz, 1972)
amylases have been purified by successive Sephadex and Bio-
Gel chromatography. This technique relies upon the affinity
of amylase for the dextran matrix of Sephadex and the
resulting slower elution of amylase. The failure of this
technique to produce electrophoretically pure amylase in the
present study may have resulted from the affinity of several
amylases or other carbohydrases for the dextran matrix.
Robyt et al. (1971) obtained identical molecular weight
values for porcine amylase fractionated on Sephadex and Bio-
Gel, indicating that the elution of amylase was not slowed
by an affinity for the Sephadex. The different Ve/Vo ratios
given in the present study for Sephadex gel filtration with
sodium phosphate and NaCl eluants demonstrates the effect of
different eluants on the gel filtration of amylase. Thus
Robyt et al. (1971), by using a pH 8.5 eluant, may have
reduced the amylase activity and consequently the Sephadex-
amylase interaction.

Reported molecular weights of invertebrate and mam-
malian amylases have ranged from 40,000 to 60,000 (Fischer
and Stein, 1960; Frati and Caputo, 1970; Cozzone et al., 1970;
Robyt et al., 1971; Wojtowicz and Brockerhoff, 1972; Wojto-
wicz, 1972). Robyt et al. (1971) separated porcine pancrea-
tic amylase (50,000 daltons) into two subunits of 25,000 dal-
tons by strong reducing conditions which presumably broke
disulfide bonds. They also gave some evidence of 12,500-
dalton subunits. Thus the low molecular weight amylases of

peak B in the present study probably correspond to subunits of the porcine amylase. Although the low molecular weight amylases probably associate with each other or with style-matrix proteins eluted at peak A, they are not bound together by strong disulfide bridges as are the porcine amylase subunits.

Agarose and polyacrylamide electrophoresis demonstrate the high degree of protein heterogeneity in the style. Three different amylases were demonstrated by agarose electrophoresis. The large number of amylase bands seen by polyacrylamide electrophoresis may result from partial dissociation of the amylases.

Although style amylase is similar to mammalian amylase in several respects as discussed above, differences demonstrated by gel filtration chromatography are additionally indicated by our findings that antibodies against porcine pancreatic amylase would not precipitate any proteins from whole style homogenate.

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