Tanning in the Spermatophore of a Crustacean (*Penaeus trisulcatus*)

It now seems established that tanned structures are of widespread occurrence among the invertebrates[1,2]. A hitherto unsuspected structure, the spermatophore of a penaeid prawn, is here reported to owe its chemical stability to an enzymically-catalyzed tanning process, but one in which no free dihydroxyphenols are involved.

Sections through the proximal region of the vas deferens of *Penaeus trisulcatus* reveal a highly viscous fluid to mix, at first, with the sperms incoming from the testis. The sperm mass thus produced next acquires two covering layers (I and II) in the succeeding main region. They give rise to a cylindrical main body in but one duct, when in an accompanying wing duct of this region 3 other secretions (III, IV and V) simultaneously contribute to the formation of an accessory solid wing. No sooner do these ducts become confluent with one another than secretion V from the wing establishes a preliminary, but weak, connexion with the body after it has set down around its outer surface.

Important changes take place in the terminal ampoule. The tubular body is moulded into a globular form and the wing spread out into an extensive flattened sheet. A further yield of secretion III furnishes another, but more firm, connexion between these 2 parts. And lastly, protective layers, VI around the body and VII on both surfaces of the wing, are added.

Until this stage of formation the entire spermatophore is to be regarded as soft. Its conversion into a hard structure, initiated in the ampoule itself at the ontogenetically correct time, is believed to continue well after it has been transferred to the female. In agreement with observations on other tanned structures[3], only those layers (II, IV and V) that proved to consist of lipoprotein are involved in this change. It is interesting that the lipid, shown by the Liebermann-Burchardt test to be of steroid type, loses its ability to stain shortly before tanning is initiated, presumably as a result of polymerization. The protein moiety, on the other hand, is characteristically rich in phenolic groups owing to the presence of tyrosine. No free phenols could, however, be detected either histochemically or after prolonged extraction with suitable solvents, but a phenolase, readily demonstrable by the 'Nadi' reagent, does exist. If sections of soft spermatophores, before the onset of the tanning process, are incubated with catechol under suitable conditions pronounced darkening takes place, indicating that the existing phenolase is capable of promoting active oxidation of phenolic substrates. Since this effect is not produced in previously boiled sections or after treatment with cyanide, it is concluded that this enzyme complex, restricted as it is to the layers destined to be tanned, eventually oxidizes the tyrosyl residues of the naturally occurring substrate in situ, in the manner originally suggested by BROWN[4] and later shown to be chemically possible by HACKMAN[5].

Although the tanned layers fail to darken appreciably in colour, they strongly reduce ammoniacal silver hydroxide, undergo pronounced changes in affinity for stains and in isoelectric point, and develop a greater resistance to acids. All of these criteria, associated with tanning[1], clearly indicate that the crustacean spermatophore may owe its hardness, not to the mere exposure to sea water as has hitherto been thought[6-9], but to a definite enzymically-catalyzed chemical transformation. It is suggested, in conclusion, that this hardness, likewise existing in forms other than *Penaeus*, is the cause of the difficulty reported by earlier workers in sectioning spermatophores[9,10]. A full account of these and other aspects of the present work will be published elsewhere.

Résumé. Le spermatophore chez *Penaeus trisulcatus* (Crustacés Décapodes) a une structure très compliquée. Sa dureté est dûe à un tannage phénolique qui ressemble à ce qui a lieu en général dans les cuticules d'arthropodes, bien qu'il n'implique pas de polyphénols libres.

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Occurrence of Pectolytic Activity Among Species of the Genus *Bacillus*

The degradation of pectin by micro-organisms has engaged scientists largely because of its relation to phytopathogenesis[1-3], its role in softening stored fruits[4] or fermented olives[5] and because of its significance in retting plant fibres[6-8]. Biochemically, the retting of plant fibres from flax, hemp and jute stems is nothing more than the maceration of pectinous cell material to obtain clean fibres.

Pectolytic enzymes are found among fungi[1,9,10], yeasts[5], actinomycetes[11,12] and various types of bacteria[1,11,13]. Nevertheless, in the case of retting, a specific pectolytic flora, for the greater part consisting of aerobic and anaerobic spore-forming bacteria, has been claimed responsible[6]. Although pectolytic activity has been observed with some species of the genus *Bacillus*[14-16], neither the distribution of this property among the common soil-inhabiting *Bacillus* species, nor its use as a taxonomic aid in classifying unknown spore-forming bacilli has been studied so far.

Method. Using the gel liquefaction technique, a total of 99 different strains belonging to 18 different recognized *Bacillus* species, was examined for ability to degrade pectin. The following basal medium was found suitable. Peptone (Merck), 5.0 g meat extract (Merck), 3.0 g; yeast extract (Difco), 0.5 g; glucose, 0.5 g; NaCl, 0.1 g and $CaCl_2 \cdot 2H_2O$, 3.0 g; distilled water, 1000 ml; agar (Oxoid No. 1), 10.0 g/l. Before autoclaving, the pH of the basal medium was adjusted to 6.0, 7.0 and 8.0,

Differentiation of pectin-degrading, aerobic spore-forming bacilli isolated from natural environment

Species	Gas from glucose	Amylase	$NO_3^- \rightarrow NO_2^-$	Gas from NO_3^-	Lecithinase
B. macerans-polymyxa	+	+	+	var.	var.
B. licheniformis	—	+	+	+	—
B. pumilus	—	—	—	—	—
B. subtilis	—	+	+	—	—
B. pantothenticus	—	+	var.	—	+

respectively, using a glass electrode. A sterile 2% pectin suspension (final pH = 4.5) was poured on top of the basal medium and left overnight to give a firm gel. The final pH of the pectin layer was estimated with indicator paperstrips soaked in distilled water. The following pH ranges were measured on a basal agar of pH 6, 7 and 8, respectively: range I: 5.3–5.5; range II: 6.7–6.9; range III: 7.4–7.6.

The preparation of the 2% pectin suspension should be made carefully in order to prevent clumping. For 1 l basal medium, 10 g pectin were suspended in approximately 15 ml ethanol (95%) and this suspension was transferred to 500 ml distilled water while stirring. Sterilization was restricted to 1 min only (at 121°C), as the gelling ability decreased with increasing autoclaving time. As pectin, a low methoxylated apple pectin ('Rotband Pektin') from Obipektin AG, Bischoffzell, Switzerland, was used.

Origin of cultures. Several cultures of 18 *Bacillus* species were obtained partly from the Czechoslovak Collection of Micro-organisms, Brno, and partly from the Sammlung für Mikroorganismen, Göttingen. In addition, a number of strains collected by the author were used. The organisms were kept on nutrient agar and cross-inoculated on the pectin layer. Plates were observed for a) liquefaction and/or b) a furrow-like indentation of the pectin gel after 2, 4 and 7 days (28°C). Strains of *B. coagulans* and *B. steorothermophilus* were grown at 50°C, *B. pantothenticus* at 37°C.

Number of strains tested. B. alvei (8), *B. brevis* (3), *B. circulans* (5), *B. coagulans* (2), *B. cereus* (8), *B. cereus* var. *mycoides* (7), *B. firmus* (2), *B. lentus* (2), *B. licheniformis* (7), *B. macerans* (3), *B. megaterium* (8), *B. pantothenticus* (1), *B. polymyxa* (11), *B. pumilus* (8), *B. sphaericus* (6), *B. subtilis* (9), *B. stearothermophilus* (2) and *B. thuringiensis* (7).

Results and discussion. Among the 99 strains tested, pectolytic activity appeared to be restricted to 6 species belonging to 3 groups: 1. the *B. macerans-polymyxa* group, 2. the *B. licheniformis-pumilus-subtilis* group and 3. to *B. pantothenticus*. All strains of the species *macerans, polymyxa* and *pantothenticus* showed complete liquefaction of the pectin layer within 4 days. Liquefaction was apparent throughout the 3 pH levels tested, though the process was most rapid in alcaline and acid pectin layer. On the other hand, only part of the strains belonging to the *licheniformis-pumilus-subtilis* group was equipped with pectinases, as 2 strains of each species revealed a negative test. Pectolytic strains of *B. subtilis* and *B. pumilus* gave the strongest activity in the alcaline pH range (pH 7.4–7.6), whereas those of *B. licheniformis* were most markedly positive in the acid environment (pH 5.3–5.5). Of these 3 species, a weak liquefaction was observed only with the *B. subtilis* strains.

From these results three main conclusions may be drawn. First, the differences in pH optimum for pectin degradation suggest the involvement of different enzymes or complexes of enzymes. This is not surprising, since at least 2 hydrolytic pectic enzymes and one nonhydrolytic breakdown or *trans*-eliminative split of pectin has been described for several micro-organisms[13,15,17]. Secondly, it can be assumed that such wide-spread bacilli as *B. cereus, B. cereus* var. *mycoides, B. circulans* and *B. megaterium* are of no importance as members of a subsidiary pectolytic flora macerating stems and attacking fresh litter. In retrospect, the restricted distribution of pectinases among aerobic spore-forming bacilli could be expected, since only *B. subtilis, B. macerans* and *B. polymyxa* were isolated from retting material[6] and *B. licheniformis* (as well as 2 unidentified *Bacillus* sp.) as the only pectolytic bacilli from the rhizosphere[12]. Obviously, only a restricted number of soil *Bacillus* sp. should be regarded as important in degrading pectinous substances.

Finally, the restricted number of pectin degrading *Bacillus* species appears as a useful aid in their classification. Thus, with the aid of only a few additional physiological tests[18], such pectin-degrading aerobic spore-forming bacilli may be classified rapidly.

Zusammenfassung. In einer vergleichenden Untersuchung wurden 99 Stämme aus 18 Arten der Gattung *Bacillus* auf die Bildung von Pectinasen mit dem Plattentest geprüft. Das begrenzte Vorkommen der pektinzersetzenden Enzyme unter den *Bacillus*-Arten lässt darauf schliessen, dass diese Gruppe von Organismen beim ersten Angriff auf den wichtigsten Bestandteil pflanzlicher Gewebe nur eine untergeordnete Rolle spielt (saprophytisch oder parasitisch).

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