Naturally Occurring Low-Molecular-Weight Peptides from the Blowfly *Phormia regina*

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Freely occurring α -L-aspartyl-L-histidine, β -alanyl-L-histidine (carnosine), α -L-aspartyl- α -L-lysine, L-asparaginyl- α -L-lysine, α -L-glutamyl- α -L-lysine, L-isoleucyl- α -L-lysine, L-seryl- α -L-lysine, L-valyl- α -L-lysine and glycyl- α -L-lysyl- α -L-lysine were identified in young larvae (second and third instars) of the blowfly (*Phormia regina*).

The haemolymph of the blowfly (*Phormia regina*) in the early third instar contains a minimum of nineteen small soluble peptides. The amino acid composition of nine of the major peptides has been determined; eight of these are dipeptides and one is a tripeptide. The remaining ten occur in low concentrations and have not been investigated. The maximum concentration of peptides occurs in the early third instar and decreases rapidly thereafter, the fully grown larva being virtually devoid of these peptides (Levenbook, 1966). The present paper deals with the amino acid sequence of the major transitory peptides.

MATERIALS AND METHODS

Isolation of peptides. Blowfly larvae were grown as described by Levenbook & Dinamarca (1966). Batches of 5–25 g. of larvae in late second–early third instars were homogenized in 9 vol. of 3% (w/v) sulphasalicylic acid, the precipitated protein was removed by centrifugation and washed with 1% (w/v) sulphasalicylic acid, and the combined supernatants were desalted on a Dowex 50 column (H^+ form). Amino acids and peptides were eluted with aq. 4N- NH_3 soln., and NH_3 was removed on a rotary evaporator. The residue was dissolved in 0.01 M-collidine-acetate buffer, pH 8.0, and shaken with an excess of cupric carbonate. The resulting mixture of copper complexes of amino acids and peptides was resolved into five fractions on a DEAE-cellulose column by the method of Tommel, Vliegenthart, Penders & Arens (1966).

Cu^{2+} was removed from each fraction by shaking it with 8-hydroxyquinoline in chloroform, and the fractions and their hydrolysates were examined by high-voltage ionophoresis at pH 1.9, 3.7 or 6.4 (Dreyer & Bynum, 1967). Peptides were located and subsequently isolated by preparative ionophoresis. The composition and purity of peptides were established by ionophoresis of their acid hydrolysates or enzymic digestion products. When

necessary, impure samples were eluted from the paper, concentrated and reisolated at a different pH.

N-Terminal amino acids. These were identified by the 'dansylation' procedure of Gray & Hartley (1963) and Gray (1967).

Enzymic hydrolysis. Peptides were hydrolysed with diisopropyl phosphorofluoridate-pretreated LAP* (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) as described by Light (1967).

Reference peptides. Synthetic dipeptides were purchased from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A., and re-purified by ionophoresis.

RESULTS

Results of complete acid hydrolysis with 6N-hydrochloric acid for 18 hr. at 112°, partial hydrolysis with 0.25N-acetic acid for 18 hr. at 100° for aspartyl bonds (Hill, 1965), LAP digestion, 'dansylation', and ionophoretic co-mobility with known peptides usually allowed an unambiguous assignment of amino acid sequence (Table 1). The type of linkage involved, i.e. α - or β -carboxyl of aspartic acid, α - or γ -carboxyl of glutamic acid and α - or ϵ -amino of lysine, is also apparent from Table 1. The cleavage of peptides with LAP strongly suggests that only L-amino acids were involved. D-Amino acids have not been detected in *P. regina* (Levenbook & Dinamarca, 1966). There are additional arguments concerning the type of linkage involved. Thus, ϵ -amino linkages in lysine-containing peptides were ruled out because of the formation of DNS- ϵ -lysine in all cases. α -Carboxyl linkage in aspartic acid-containing peptides (nos. 7 and 8) was decided on the basis of their hydrolysis with LAP; β -linked aspartic acid residues are not

* Abbreviations: LAP, leucine aminopeptidase; DNS, 1-dimethylaminonaphthalene-5-sulphonyl.

Table 1. *Amino acid sequence analysis of blowfly peptides*

Peptide no.*	Ionophoretic mobility at pH 6.4	Products of hydrolysis				Proposed structure
		6N-HCl, 112°, 18hr.	0.25N-Acetic acid, 100°, 18hr.	LAP, 37°, 2 or 12hr.	DNS chloride products	
7	Neutral	Asp, His	Asp, His	Asp, His (12hr.)	DNS-Asp	α -L-Asp-L-His
8	Neutral	Asp, Lys	Asp, Lys	Asp, Lys (12hr.)	DNS-Asp, DNS- ϵ -Lys	α -L-Asp- α -L-Lys
10	Neutral	Glu, Lys	—	Glu, Lys (2hr.)	DNS-Glu, DNS- ϵ -Lys	α -L-Glu- α -L-Lys
12	Basic	β -Ala, His	—	—	DNS- β -Ala	β -Ala-L-His (carnosine)
14	Basic	Ser, Lys	—	Ser, Lys (2hr.)	DNS-Ser, DNS- ϵ -Lys	L-Ser- α -L-Lys
16	Basic	Asp, Lys	—	Asn, Lys (2hr.)	DNS-Asp, DNS- ϵ -Lys	L-Asn- α -L-Lys
17	Basic	Gly, Lys (1:2)	—	Gly, Lys (1:2) (2hr.)	DNS-Gly, DNS- ϵ -Lys (1:2)	Gly- α -L-Lys- α -L-Lys
18	Basic	Val, Lys	—	Val, Lys (2hr.)	DNS-Val, DNS- ϵ -Lys	L-Val- α -L-Lys
19	Basic	Ile, Lys	—	Ile, Lys (2hr.)	DNS-Ile, DNS- ϵ -Lys	L-Ile- α -L-Lys

* Peptide nos. refer to the order in which the peptides are eluted from a Dowex 50 column (Levenbook, 1966)

released by this enzyme (Light, 1967). In addition, peptide no. 7 gave a 'normal' purple colour with ninhydrin, whereas it is known that β -L-aspartyl-L-histidine gives a yellow colour (Kakimoto & Armstrong, 1961). Peptide no. 8 had identical mobility with authentic α -L-aspartyl- α -L-lysine at pH 1.9 and 3.7. α -Carboxyl linkage in the glutamic acid-containing peptide (no. 10) was decided from the co-mobility of this peptide with authentic α -L-glutamyl- α -L-lysine at pH 1.9 and 3.7. Co-mobility in this instance is most significant, since at pH 4 γ -glutamyl di- and tri-peptides have mobilities over twice as high as the α -linked ones (Waley, 1966).

Peptides nos. 8 and 16 were given additional consideration since both yielded aspartic acid and lysine on strong acid hydrolysis. The fact that no. 8 was neutral whereas no. 16 was basic suggested the possibility of *N*-terminal asparagine in the latter. Incubation of no. 16 with LAP yielded lysine and a second spot that showed co-mobility at pH 1.9 with authentic asparagine and also gave a brown colour with ninhydrin, characteristic of asparagine. The presumed asparagine spot when reisolated and treated with purified guinea-pig serum asparaginase (Tower, 1967) yielded aspartic acid, establishing beyond reasonable doubt that asparagine was indeed present in the original peptide. This result is noteworthy since free asparagine was undetectable in *P. regina* (Levenbook & Dinamarca, 1966). On the other hand, although glutamine is the most abundant free amino acid in blowfly haemolymph (Levenbook, 1966), no glutamyl peptides have as yet been detected.

Possible cyclization of di- and tri-peptides during isolation and purification was observed only in one instance. When either blowfly α -L-glutamyl- α -L-

lysine (peptide no. 10) or the synthetic product was eluted from pH 3.7 ionograms, evaporated to dryness on a rotary evaporator at a temperature not exceeding 50°, and re-run at pH 3.7, small amounts of a second spot were invariably generated. This material was less basic than the parent compound, yielded only DNS- ϵ -lysine on 'dansylation', and could be converted into α -L-glutamyl- α -L-lysine by heating with 2N-hydrochloric acid at 100° for 1 hr. The derived compound was unaffected by trypsin (Worthington Biochemical Corp.) and hence was probably not a diketopiperazine. Treatment with carboxypeptidase B (Worthington Biochemical Corp.) yielded free lysine and a ninhydrin-negative, starch-potassium iodide-positive spot with a mobility similar to that of authentic pyrrolidone-carboxylic acid. The starch-potassium iodide-positive material on hydrolysis with 2N-hydrochloric acid at 100° for 1 hr. gave glutamic acid. These observations are all consistent with the derived material being pyroglutamyl-lysine.

DISCUSSION

The biological significance of this transitory group of blowfly peptides is completely unknown at present. They are not unique to *P. regina*, since we have detected several of them in extracts of larvae of the fleshfly *Sarcophaga bullata* and the housefly *Musca domestica* in the late second-early third instars. It is very unlikely that they are mere digestive products from the diet, since peptides of the type described occur in similar amounts in *P. regina* reared on meat or a sterile casein diet (Levenbook, 1966). Labelled peptides were detected in *P. regina* larvae reared on a chemically semi-defined diet (cf. Levenbook & Dinamarca, 1966)

containing uniformly labelled *Chlorella* protein hydrolysate, indicating synthesis from free amino acids.

Either histidine or lysine is the *C*-terminal residue of each of the peptides examined, thus establishing a well-defined pattern of sequence. Although the significance of this observation is unknown, it is tempting to suggest a common mode of synthesis for this group of fly peptides. In this connexion, Kalyankar & Meister (1959) found that chick pectoral-muscle carnosine synthetase catalyses the formation of carnosine as well as anserine, homocarnosine and ophidine. Products were also formed when β -alanine was replaced by several other β - or ω -acids, or when histidine was replaced by lysine, ornithine, arginine and several other amino acids. A similar enzyme(s) of such broad specificity may exist in *P. regina* larvae in the late second-early third instars.

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