

INSULIN-LIKE AND GLUCAGON-LIKE PEPTIDES IN INSECT HEMOLYMPH

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Abstract—Haemolymph from the tobacco hornworm, *Manduca sexta* (L.), was examined for insulin-like, glucagon-like and gastrin-like peptides. Several immunoreactive components similar in size to vertebrate insulin and glucagon were separated by gel filtration of acid extracts of larval and pupal haemolymph. No gastrin-like peptide was detected. These results provide evidence that at least two of these peptide hormones are transported in haemolymph to target tissues in insects.

Key Word Index: Tobacco hornworm, *Manduca sexta*, insulin, glucagon, haemolymph, radioimmunoassay

INTRODUCTION

NEUROENDOCRINE tissues of adult tobacco hornworm, *Manduca sexta* (L.) contain insulin-like, glucagon-like and gastrin-like polypeptides (TAGER *et al.*, 1975, 1976; KRAMER *et al.*, 1977a). The first two peptides modulate trehalose and glycogen levels when injected into the larval form of the same species. No physiological activity has yet been identified for the gastrin-like peptide. If these peptides are indeed hormones, then they should be transported in the circulatory system to target tissues. In this report, we demonstrate the presence of insulin-like and glucagon-like peptides in haemolymph from larvae and pupae of *M. sexta*. No immunoreactive gastrin was detected.

MATERIALS AND METHODS

Insects

Eggs of *Manduca sexta* were a gift from Dr. J. REINECKE (Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Fargo, ND, U.S.A.). Larvae were reared at 28°C and 60% r.h. during a 16 hr light–8 hr dark photoperiod on a standard diet (BELL and JOACHIM, 1976).

Haemolymph collection.

Fifth instar larvae and pupae were anaesthetized by cooling to 4°C before bleeding. Haemolymph was collected from larvae by cutting off the abdominal horn at its base and from pupae by cutting off the proboscis. Haemolymph was drained into a centrifuge tube containing a trace amount of 1-phenyl-2-thiourea to inhibit phenol oxidase activity, and then frozen, lyophilized, and stored at –20°C.

Preparation of extract

Procedure 1. Two grams of lyophilized haemolymph (obtained from 75 to 100 ml of whole haemolymph) were mixed with 30 ml 3 M acetic acid. The suspension was

homogenized for 1 min, incubated at 4°C for 1 hr, and centrifuged at 17,000 *g* for 20 min. at 4°C. After collecting the supernatant, the pellet was re-extracted with 15 ml 3 M acetic acid. The combined extracts were dried under vacuum.

Procedure 2. Two grams of lyophilized haemolymph were homogenized in 3.0 ml of 0.1 M HCl prepared in 60% (v/v) ethanol. The homogenate was centrifuged at 10,000 *g* for 10 min at 4°C and the supernatant was collected and adjusted to pH 6 using 6 M Na₂OH. The extract was then diluted with two volumes of ethanol and four volumes of diethyl ether. The mixture was incubated at 4°C for 18 hr and the precipitate that formed was collected by centrifugation and dried under vacuum.

Column chromatography and radioimmunoassays

Dried extracts were dissolved in 5–15 ml of 3 M acetic acid and applied to a 2.0 cm i.d. × 80 cm Biogel P-30† (Bio Rad Laboratories, Richmond, CA, U.S.A.) column previously equilibrated with 3 M acetic acid. Three-ml fractions were collected. The absorbance profile at 280 nm was obtained using a Cary 118C spectrophotometer. Samples were then pooled, dried under vacuum to remove solvent and subjected to insulin, glucagon, or gastrin radioimmunoassay using standard kits (Radioassay Systems Laboratories, Inc., Carson, CA, U.S.A.). Levels of peptides were determined graphically from standard curves constructed with results obtained from vertebrate peptide standards. The porcine insulin antiserum was produced in guinea pigs and is used in clinical laboratories routinely to measure human, dog, and porcine insulins. The porcine glucagon antiserum was raised in rabbits and is used to measure porcine, bovine and human pancreatic glucagon but not gut glucagon. The human gastrin I antiserum was produced in rabbits and is used to measure human gastrin. Lower limits of detection for insulin, glucagon and gastrin from the above species are 200, 25, and 25 × 10⁻¹² g per ml respectively.

RESULTS AND DISCUSSION

In recent years, considerable attention has been paid to the characterization of haemolymph proteins of apparent molecular weight larger than 10⁴ daltons (WYATT and PAM, 1978). Serum polypeptides of lower molecular weight appear to have been largely ignored, probably because of the earlier lack of analytical

†Mention of a proprietary product does not constitute an endorsement by the U.S. Department of Agriculture over others that may be suitable.

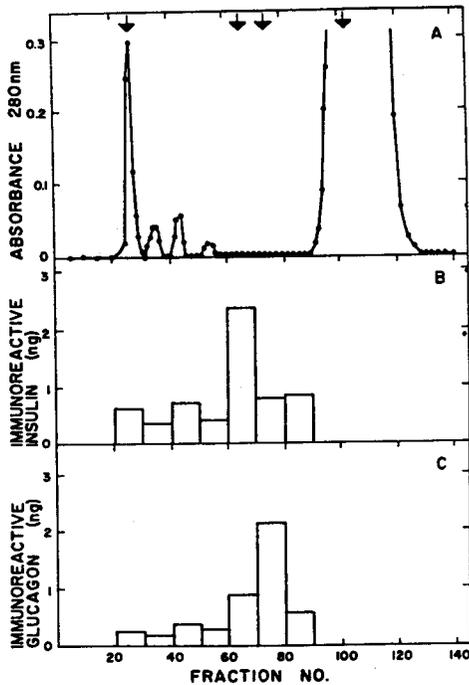


Fig. 1. Profiles of absorbance at 280 nm (A), insulin-like (B) and glucagon-like (C) immunoreactivities obtained after gel filtration of an acid extract of haemolymph from *M. sexta* larvae. The elution positions of standards from the same column are indicated by the vertical arrows which represent, from left to right, bovine serum albumin, bovine insulin, porcine glucagon and [^{14}C]-leucine, respectively.

procedures for detecting minute amounts of these substances in the presence of large amounts of other components. With the advent of the radioimmunoassay technique (BERSON and YALOW, 1973), it became possible to detect biologically active substances in the nano-picomolar concentration range. We have used radioimmunoassay to detect insulin-like and glucagon-like peptides in haemolymph of the tobacco hornworm.

If peptides present in the neuroendocrine system of insects are true hormones, then they should also be secreted into haemolymph where they can be transported to peripheral tissues. When *M. sexta* haemolymph was extracted according to procedure 1, and subjected to gel filtration on a polyacrylamide matrix, both insulin-like and glucagon-like peptides were detected by radioimmunoassay (Fig. 1). The void volume of the column occurred at fraction 27 and the inclusion volume at fraction 103. The high absorbance seen in the inclusion volume part of the profile is due to an unidentified yellow pigment present in the sample. Typical radioimmunoassay standard curves are shown in Figs. 2 and 3 for insulin and glucagon, respectively. For example, analyses of pooled samples from column fractions 81-90 and 31-40 revealed approx. 0.9×10^{-9} g insulin-like peptide and 0.2×10^{-9} g glucagon-like peptide per ml, respectively. As shown in Fig. 1B, radioimmunoassay using the insulin antiserum revealed the major component centred at fractions 60-69. Assay using the glucagon antiserum demonstrated the major component at fractions 70-79. Application of peptide standards to the same column indicated that these immunoreactive peptides have apparent molecular weights of approx. 5000 and 3000 respectively, essentially the same as the vertebrate peptides. No immunoreactivity was recovered when extraction procedure 2 was used. Both of these procedures have been utilized previously with vertebrate peptides. Apparently, insect and vertebrate peptides differ enough in solubility that the organic solvent precipitation technique (procedure 2) was not effective for insect peptides.

Under most circumstances, proinsulin is present in relatively small amounts in vertebrate plasma (STARR and RUBENSTEIN, 1974). The degree of heterogeneity suggested by small amounts of high molecular weight immunoreactive material present in the insect haemolymph extract also suggests that precursor forms of the peptides are present. The total amounts of immunoreactive insulin and glucagon detected in larval haemolymph varied over about a ten-fold

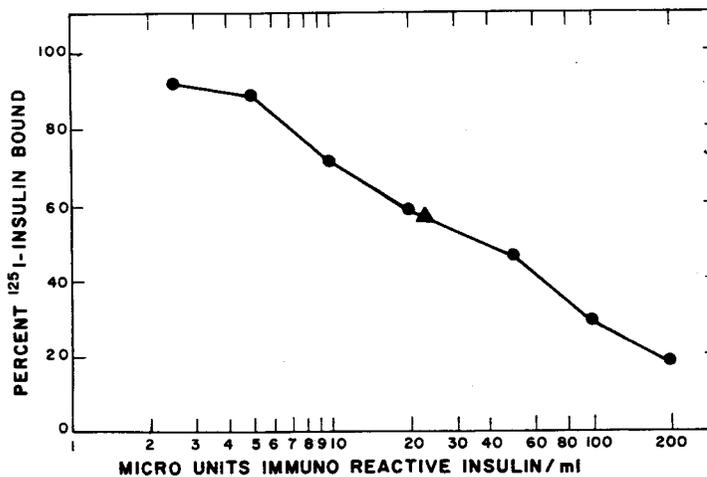


Fig. 2. Insulin radioimmunoassay competition curve. Percent iodinated porcine insulin bound versus porcine insulin standards present ranging from 2.5 to 200 μ i.u./ml (circles). By convention, one milligram porcine insulin equals 24 i.u. Triangle = pooled fractions 81-90 (see Fig. 1B) from BioGel P-30 chromatography of *M. sexta* larval haemolymph.

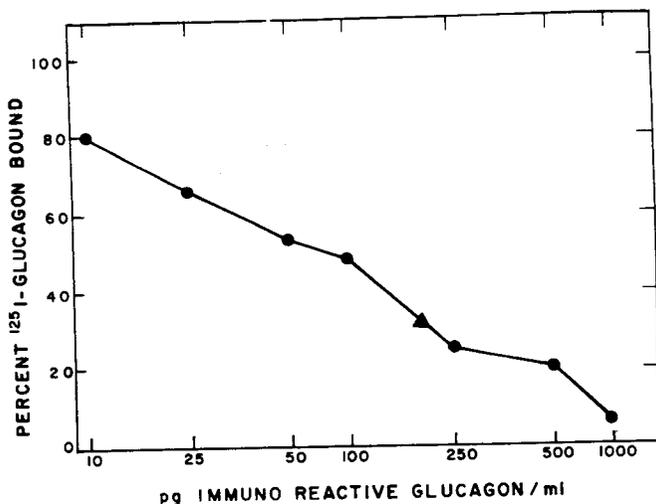


Fig. 3. Glucagon radioimmunoassay competition curve. Percent iodinated porcine glucagon bound versus porcine glucagon standards present ranging from 10 to 1000 pg/ml (circles). Triangle = pooled fractions 31-40 (see Fig. 1C) from BioGel P-30 chromatography of *M. sexta* larval haemolymph.

concentration range from $0.2-1.5 \times 10^{-12}$ g vertebrate hormone equivalents per ml, respectively. Although these quantities are somewhat less than those of typical hormone concentrations in vertebrate serum (GANONG, 1971), they are in the range that elicited hypotrehalosemia and glycogenolysis in the *M. sexta* biological assay (TAGER *et al.*, 1976). Since affinities of mammalian antibodies for insect peptides are probably less than those for vertebrate hormones, these levels should be regarded as lower limits.

Additional evidence for the macromolecular and peptide nature of the immunoreactive factors was obtained by showing that the immunoreactive peptides maintained their integrity on refractionation on Bio-Gel P-30 and exhibited a 90% loss in specific immunoreactivity when incubated with pronase (Calbiochem) for 20 min at pH 8 and 37°C. The lability of immunoreactive insulin and glucagon from insect haemolymph is in accordance with that of the vertebrate peptide.

When pupal haemolymph was extracted and subjected to radioimmunoassay, both insulin-like and glucagon-like peptides were also detected, but at reduced concentrations of approx. 0.08×10^{-12} and 0.07×10^{-12} g equivalents per ml, respectively. These levels are no doubt related in part to the separate metabolic needs of the feeding and nonfeeding forms of *M. sexta*. Although very active metabolically, pupae apparently utilize lower hormone concentrations than do larvae.

Even though we previously found that insect nervous tissue contained a peptide that is gastrin-like in its antigenicity, size and susceptibility to degradative enzymes (KRAMER *et al.*, 1977a), we were unable to detect any gastrin immunoreactivity in haemolymph extracts, at least none greater than ten picogram human gastrin equivalents per millilitre. The gastrin-like peptide found in brain may not be a hormone in the classical sense, since it appears to be confined just to neuroendocrine tissue (brain) or perhaps it is just not secreted or does not accumulate during the life stages examined here.

This study demonstrates that insect haemolymph contains peptides that compete with vertebrate insulin and glucagon in specific radioimmunoassay. These results and those of others demonstrating immunological and intraspecific biological activities of similar peptides (ASSAN *et al.*, 1969; TAGER *et al.*, 1976; KRAMER *et al.*, 1977b; MAJER *et al.*, 1978; NORMAN and DUVE, 1978) provide evidence that these peptides are indeed hormones. They are secreted by the neuroendocrine system into the haemolymph for delivery to target tissues where they affect carbohydrate metabolism. The mechanisms by which these peptides regulate sugar homeostasis are being investigated.

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