Detection of Proteins with a High Affinity for Imidazole Insect Growth Regulator, KK-42

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For explanation on mode of action of KK-42, an imidazole insect growth regulator, an amine analog of KK-42 (1-(3-aminopropyl)-5-[ (E)-2,6-dimethyl-1,5-heptadienyl]imidazole, ADHI) has been designed and prepared as a ligand to detect proteins with high affinity in pharate first larvae of wild silk moth associated with artificial itching. The mode of action of KK-42 for induction of precocious metamorphosis is speculated to be an inhibition of ecdysteroid biosynthesis at the prothoracic gland (PG). Since the PG in 5th instar larvae of silkworm on day 7 has strong biosynthetic activity and is sensitive to KK-42, a supernatant of a homogenate of the PG was applied to ADHI-Sepharose. After optimization of the condition of affinity chromatography, a single protein was eluted with free KK-42. This protein did not bind to the affinity gel when incubated with KK-42 before the application.

Keywords: KK-42, binding proteins, Bombyx mori, affinity purification.

INTRODUCTION

An imidazole compound, KK-42 (1 in Fig. 1) is an insect growth regulator (IGR) that has been known to cause precocious metamorphosis in the silkworm Bombyx mori, the European corn borer Ostrinia nubilalis, and the flesh fly Neobellia bivittata. The compound has also been reported to have other biological effects, such as the breakage of egg-diapause in the Japanese oak silkworm Antheraea yamamai and the gypsy moth Lymantria dispar japonica, as well as the induction of abnormal metamorphosis in B. mori, the locust Locusta migratoria, and the Hawaiian cockroach Dioplopleura punctata.

Originally, it was thought that KK-42 acted as an anti-juvenile hormone (JH) in B. mori. It has been revealed, however, that KK-42 inhibits the biosynthesis of ecdysteroids in prothoracic glands (PGs) both in vitro and in vivo. Currently, it is predicted that this inhibitory effect reduces the titer of ecdysteroids in hemolymph, which in turn inactivates the biosynthesis of JH in the corpora allata and induces the activity of hemolymph JH esterase. Subsequently, the JH titer in hemolymph is decreased, inducing precocious metamorphosis. In addition to the PG, several other organs may be affected by KK-42, which is associated with a variety of biological effects. To detect the site(s) having high affinity for KK-42 in diapausing pharate first instar larvae of A. yamamai, an amine analog of KK-42, 1-(3-aminopropyl)-5-[ (E)-2,6-dimethyl-1,5-heptadienyl]imidazole, ADHI, 2 in Fig. 1) was prepared for coupling with Sepharose (ADHI-Sepharose, 3), and a putative binding protein was obtained. In this study, we used the ADHI-Sepharose to purify a protein having affinity with KK-42 from the PG of B. mori.

MATERIALS AND METHODS

1. Chemicals

1-(3-Aminopropyl)-5-[ (E)-2,6-dimethyl-1,5-heptadienyl]imidazole (ADHI, 2) and ADHI-Sepharose (3) were prepared in a previous study to obtain binding protein from diapausing pharate first instar larvae of A. yamamai.

2. Insect and Tissue Preparations

Silkworms, B. mori (C140 × N145), were reared on an artificial diet including mulberry leaves at 25°C with a photoperiod of 12L/12D. The prothoracic glands (PGs) of the larvae at different stages were dissected in 0.75% NaCl solution.

3. Affinity Chromatography

One hundred pairs of silkworm PGs from 5th larvae on day 7 were homogenized in 1 ml of purification buffer (20 mM Tris-HCl, pH 7.6, containing 1% SDS, 5 mM magnesium acetate, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml of leupeptin, and 1 μg/ml of pepstatin A). The homogenate was then sonicated and centrifuged at 10,000 × g for 10 min. The supernatant fractions were used after filtration as a starting crude protein preparation for the affinity purification. Briefly, the ADHI-affinity gel was packed into a column with a cap and glass-frit
base and washed thoroughly with the purification buffer. The crude protein preparation (approx. 0.1 mg/ml) was added to the affinity gel and shaken gently in the column for 16 hr. After loading, the gel was washed 5 times with gentle shaking using 10 bed volumes of purification buffer and the purification buffer containing 0.1 mM farnesol as an optimized washing solution. Then, a ligand-specific elution was carried out with gentle shaking using a 0.1 M KK-42 solution (0.1 mM in the purification buffer). For competition experiments, the crude protein preparation was incubated with 10 mM KK-42 in the purification buffer for 16 hr at 4°C prior to its loading onto the affinity column.

4. Polyacrylamide Gel Electrophoresis (PAGE) Analysis

The crude protein preparation and fractions from the affinity chromatography were analyzed by SDS-PAGE and visualized with silver staining.

RESULTS AND DISCUSSION

Since an isoprenyl chain and an imidazole ring are characteristic moieties in KK-42 (1), an amine possessing these structures, ADHI (2) in Fig. 1, was designed and prepared as a ligand for the affinity matrix. The amine was then coupled with CNBr-activated Sepharose to afford ADHI-Sepharose (3). As a target molecule of KK-42, we obtained a affinity chromatography with the ADHI-Sepharose. This method was used to identify target molecule(s) in the B. mori PG which are associated with biological events, such as the induction of precocious metamorphosis. A homogenate of PGs prepared from the ultimate larvae on day 7 in Tris-HCl buffer was used as the starting material for the affinity purification. The PG has strong ecdysteroid synthesizing activity, and so the organ is very sensitive to KK-42. Since almost all of the protein in the supernatant was bound to the gel (data not shown), both the binding and eluting conditions were optimized. The addition of 1% SDS prevented most non-specific binding. Although washing and eluting were carried out under several conditions, such as by supplementing the washing buffer (1% SDS in Tris-HCl buffer, pH 7.6) with 25% ethanol, 10% ethylene glycol, 2 M NaCl, or 2 M urea, no selective protein was eluted (data not shown). In contrast, some proteins could be eluted with 0.1 mM farnesol, a sesquiterpene analogous to JH, as shown in Fig. 2. This result suggests the presence of proteins with a high affinity for farnesol in the B. mori PG. These proteins might be involved in the recognition of farnesylated proteins during protein-protein interaction, such as small G-proteins. Then, we succeeded in eluting a protein having specific affinity from the ligand by adding 0.1 mM KK-42 to the buffer after the farnesol elution. As this protein was larger than the markers used in the 20% SDS-PAGE, the analysis was repeated on a 5% gel, and the binding protein was estimated to have a molecular mass of approximately 220 kDa (Fig. 3). This protein did not bind to the affinity gel when the PG homogenate was pre-incubated in the presence of KK-42.

In a previous study, we obtained a 45 kDa KK-42-binding protein from the pharate first instar larvae of A. yamamai with ADHI-affinity gel; this bound protein was associated with artificial hatching (break of diapause). The KK-42-binding protein obtained from the B. mori PG in the present study was quite dif-

![Fig. 2. SDS-PAGE analysis (10% gel) of fractions of PG proteins obtained by affinity chromatography with ADHI-Sepharose. M: molecular weight markers, C: crude preparation, nB: non-binding fraction, S: fraction eluted by washing with 1% SDS, F: fraction eluted with farnesol, K: fraction finally eluted with KK-42. (+)(−): samples incubated with or without KK-42 prior to the affinity purification.](image)
different from that in A. yamamai in terms of molecular mass. The 220 kDa protein with high affinity for the ADHI-gel might be a candidate for a target molecule involved in the induction of precocious metamorphosis. It may be reasonable to assume that KK-42 binds to several different molecular species given that it has a variety of biological effects. We have found that topical application of KK-42 at day 1 of the 4th stadium induced the enzymatic activity of hemolymph JH esterase (JHE).\(^\text{10}\) JH and JH analogs also induce JHE activity when applied to final instar larvae of cabbage looper Trichoplusia ni\(^\text{9}\) and tobacco budworm Heliothis virescens;\(^\text{5}\) therefore, the possibility that KK-42 affects proteins related to the regulation of JH cannot be excluded. A larger amount of the 220 kDa binding protein needs to be purified for further characterization.

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