Comparative amidolytic activity of nattokinase with several synthetic substrates

Substrate	Substrate hydrolysis (nmol/min/ml)
H-D-Val-Leu-Lys-pNA (S-2251)	68.5
Bz-DL-Arg-pNA	18.0
H-D-Phe-Pip-Arg-pNA (S-2238)	14.0
H-D-Val-Leu-Arg-pNA (S-2266)	13.5
H-D-Pro-Phe-Arg-pNA (S-2302)	11.5
pyro-Glu-Gly-Arg-pNA (S-2444)	0
pyro-Glu-Pro-Val-pNA (S-2484)	0

The reaction mixture (1 ml) contained 20 µl of nattokinase extract (420 µg protein), 5×10^{-4} M substrate and 0.1 M phosphate buffer, pH 7.4. After incubation for 10 min at 37 °C, the p-nitroaniline liberated was determined from the absorption at 405 nm. The results are expressed as nmoles of substrate hydrolyzed per min per ml of nattokinase extract. Each value is the mean of 3 determinations.

was performed with a column (1.0 × 25 cm) of Sephadex G-100 (Pharmacia Chemicals) equilibrated and run with 0.1 M phosphate buffer containing 0.2 M NaCl, pH 7.4. Isoelectric focussing was carried out according to the method of Vesterberg and Svensson[11] using ampholytes of pH 3.5–10.5.

Results and discussion. A strong fibrinolytic activity was found when Natto was applied directly to the fibrin plates (fig. A). This fibrinolytic enzyme (nattokinase) could be extracted easily with saline (fig. B). The calculated fibrinolytic activity of the extract obtained from 1 g wet Natto corresponded to about 40 CU plasmin or 1600 IU urokinase, when calculated from each standard fibrinolytic enzyme. Although in a crude state, the nattokinase was relatively stable at neutral pH but was gradually inactivated above 60 °C (fig. C). After 5 rounds of freezing and thawing, more than 95% of the activity remained. At neutral and alkaline pH values (7–12) the nattokinase was stable for 10 min at room temperature, but it was labile below pH 5.0. With some additional substances such as boiled rice extract, boiled meat extract, serum albumin and gastric mucin (1–50 mg dry wt/ml), the stability of nattokinase was much increased and the enzyme activity was not completely inhibited (in any case more than 7.5% of the original activity remained) even in acidic conditions.

The activity was not altered with or without 5 mM Cys, whereas 1 mM DFP and 5 mM Neguvon completely inhibited the fibrinolysis, suggesting that nattokinase is a serine protease. In other experiments, the typical anti-plasminic agents ε-ACA and t-AMCHA[12,13] revealed no effect on the fibrinolysis under the same conditions (final concentration, 50 mM, in the sample applied to fibrin plates).

The amidolytic acivity of nattokinase was investigated with several synthetic substrates. As shown in the table, the most sensitive substrate was found to be the plasmin substrate S-2251, with much lesser effects for Bz-DL-Arg-pNA, S-2238, S-2266 and S-2302. There was virtually no activity on the urokinase substrate S-2444 or the elastase substrate S-2484.

By gel filtration on Sephadex G-100, nattokinase was found to show a mol. wt of about 20000. The active fraction further purified by isoelectric focussing also revealed a single symmetrical fibrinolysis peak with a pI of about 8.6.

In the present study, as outlined above, we first demonstrated the presence of a novel fibrinolytic enzyme, nattokinase, in Natto. The previous utilization of Natto for various purposes in folk medicine may be related to the effects of this strong enzyme. Recently, several fibrinolytic enzymes such as urokinase [14,15] and earthworm protease[16] have been shown to be effective for plasma fibrinolysis by oral administration. They are usually mixed with several stabilizing factors, such as serum albumin and gastric mucin, and enteric-coated. They are not only absorbed into the plasma through the gastrointestinal tract but also induce urokinase-like endogenous plasminogen activator in the plasma, probably from the vascular endothelium or liver[15]. Nattokinase may be an equally good protease for oral fibrinolytic therapy because of its confirmed safety for long-term intake, stability and the strong fibrinolytic activity demonstrated here. Further purification of and in vivo experiments on nattokinase are currently in progress.

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A survey of 3-dehydroretinal as a visual pigment chromophore in various species of crayfish and other freshwater crustaceans

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Summary. 3-Dehydroretinal (vitamin A_2 aldehyde) was found in the eyes of three species among 10 species of freshwater crayfish examined. Since dark-adapted eyes contained the 11-*cis* form of 3-dehydroretinal, this compound must be the chromophore of the visual pigment. 3-Dehydroretinal always coexisted with retinal (vitamin A_1 aldehyde), indicating the presence of a rhodopsin-porphyropsin visual pigment system.
Key words. 3-dehydroretinal; retinal; visual pigment; crayfish.

The visual pigment commonly found in invertebrates is rhodopsin, which has retinal as a chromophore. Recently, two new chromophores have been found in the visual pigment of invertebrates. One is 3-dehydroretinal, which was found in a

Type and averaged content of chromophore of visual pigment in freshwater crustaceans

Species		Animal (no; length)	Chromophore Type*	Amount**	Origin	Supplied by
Crayfish	*Procambarus clarkii*	(34;8–10 cm)	A_1,A_2	300	Japan	(Ref. 1)
	Procambarus clarkii	(3;8–10)	A_1,A_2	190	US (Calif.)	J. Usukura
	Procambarus clarkii	(6;10)	A_1,A_2	250	US (La.)	K. Enomoto
	Procambarus clarkii	(3;9)	A_1,A_2	190	Mexico	E. Frixione
	Cherax destructor	(5;7–10)	A_1,A_2	95	Australia	S. Stowe
	Euastacus armatus	(1;11)	A_1,A_2	140	Australia	S. Stowe
	Paranephrops planifrons	(4;6–8)	A_1	300	New Zealand	B. Meyer-Rochow
	Astacus torrentium	(2;5–7)	A_1	30	Germany	K. Vogt
	Astacus leptodactylus	(2;10–11)	A_1	180	Turkey	M. Bender
	Orconectes limosus	(3;7–8)	A_1	60	Germany	M. Bender
	Cambaroides japonicus	(4;3–7)	A_1	20	Japan	S. Hiruta
	Pacifastacus trowbridgii	(5;6–12)	A_1	250	Japan	S. Hiruta
	Pacifastacus leniusculus	(3;11)	A_1	250	Japan	M. Maehata
Others	*Macrobrachium nipponense*	(n = 3)	A_1	200		
	Palaemon paucidens	(n = 10)	A_1	100		
	Potamon dehaani	(n = 5)	A_1	25		

* A_1: Retinal, A_2: 3-Dehydroretinal. ** Averaged amount of total chromophore (pmol/eye).

freshwater crayfish[1] and a bioluminescent squid[2], and the other is 3-hydroxyretinal, found in some species of insect[3,4]. The detailed functions of these chromophores are not clear at present. Our finding of 3-dehydroretinal in the compound eyes of the crayfish *Procambarus clarkii*[1] has raised the question of whether this chromophore exists only in the eyes of *Procambarus clarkii* or is also present in the eyes of other species of crayfish and also in those of other crustacean species. In order to clarify this we have measured the visual pigment chromophore in the eyes of 10 species of crayfish which were sent to us by biologists from several countries (table) and also in the eyes of other freshwater crustaceans collected by us in Japan. Japanese *Procambarus clarkii* was first introduced from New Orleans, USA in 1930. It has previously been reported by other investigators that only retinal is present in the eyes of North American animals of this species[5,6]. For this reason, we obtained eye samples of North American populations of *Procambarus clarkii* to compare with the Japanese population.

Eyes were isolated under dim light (usually red but in some cases white) from animals usually dark-adapted for 2 days. The eyes were wrapped in aluminum foil and freeze-dried under vacuum. The dried eyes were sent, usually by air mail, from each laboratory to Suzuki's lab. The chromophore of the visual pigment was analyzed as reported previously[1,7]. Briefly, the dried eye was ground with a glass stick and washed with petroleum ether three times to remove carotenoids. The eyes of freshwater crustaceans other than crayfish were prepared under dim-red light and treated in the same way as above. Chromophore was extracted as the oxime and analyzed by HPLC (high-pressure liquid chromatography). The amounts of retinal and 3-dehydroretinal were quantified using standard curves determined with authentic compounds.

The table shows the results obtained from ten species of crayfish together some other freshwater crustaceans. The Japanese population of *Procambarus clarkii* has been repeatedly studied using a large number of animals, and there is no doubt that the animal has both retinal and 3-dehydroretinal as the chromophores of visual pigments[1,8]. All eye samples obtained from three populations of *Procambarus clarkii* in North America contained both retinal and 3-dehydroretinal, although the proportion of 3-dehydroretinal was different from individual to individual. Some previous investigators had reported that this species living in North America has only retinal-based pigment in its eye[5,6,9]. The discrepancy may be due to the labile nature of the rhodopsin-porphyropsin visual pigment system. The amount of 3-dehydroretinal rapidly decreases when crayfish are kept at higher temperatures in the presence of light[8]. Present results show that there is no difference between the Japanese population and the North American population in respect to 3-dehydroretinal. It is clear that crayfish of the species *Procambarus clarkii* have both retinal and 3-dehydroretinal as the chromophore of the visual pigment regardless of where they live.

3-Dehydroretinal was found also in the two species of Australian crayfish, *Cherax destructor* and *Euastacus armatus*. All eyes from 5 animals of *Cherax* contained both 3-dehydroretinal and retinal, most of which was in the 11-*cis* configuration. The proportion of 3-dehydroretinal varied between samples from 8 to 49% of total chromophore (average 23%). We can conclude that *Cherax destructor* has a rhodopsin-porphyropsin pigment system as *Procambarus clarkii* does. Only one individual of *Euastacus* was available because of the difficulty of collection, and the proportion of 3-dehydroretinal was low, 10%. The chromophore type of *Euastacus armatus* remains to be reconfirmed. The other 7 species of crayfish studied here have only retinal in their eyes. We studied the chromophore of 3 other species of Japanese freshwater crustaceans (the crab *Potamon* and the prawns *Palaemon* and *Macrobrachium*), but 3-dehydroretinal was not found in any species.

3-Dehydroretinal, the chromophore of porphyropsin, has been found in many species of freshwater vertebrates; teleosts, amphibians and reptiles[10,11]. The generally accepted explanation for this distribution is that porphyropsin absorbs longer wavelengths of light than the retinal-based pigment (rhodopsin) and would give a higher sensitivity in fresh water, which is normally somewhat redder than seawater[12]. The crayfish 3-dehydroretinal could then also be explained in terms of adaptation to the freshwater light environment. All species of seawater crustaceans so for examined, indeed, have only retinal as the chromophore of visual pigment[13]. In the freshwater crayfishes, however, some species have 3-dehydroretinal and others do not. In frogs and toads, generally, the larval tadpole has porphyropsin and the adult frog has rhodopsin. However, there are some exceptions; adult *Xenopus* with a highly aquatic life has prophyropsin and the *Bufo* tadpole, which lives in clear and shallow water, has only rhodopsin[10]. Adult *Rana catesbeiana* has both rhodopsin and porphyropsin, which is also explained by its aquatic habitat[14]. *Procambarus clarkii* and *Cherax destructor* live in a muddy environment, and the presence of porphyropsin may be an adaptation to their light environment. However, *Euastacus armatus* which was collected from a clear mountain stream also had 3-dehydroretinal. At present, it is not easy to

find a clear explanation for the distribution of crustacean porphyropsin (3-dehydroretinal).
There is another possible function for crayfish porphyropsin; if the porphyropsin and rhodopsin were present in different photoreceptor cells, wavelength discrimination could be possible using the two visual pigments. Intra-ommatidial distribution of 3-dehydroretinal should be investigated to evaluate this possibility.

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In vitro biosynthesis of juvenile hormone III by the corpora allata of *Calliphora vomitoria* and its role in ovarian maturation and sexual receptivity

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Summary. JH III is the only JH detected by GLC-MS in medium from in vitro incubations of corpora allata of adult females of *Calliphora vomitoria*. When corpora allata were removed from females at various times during the reproductive cycle and the JH III produced by the glands in vitro measured by a JH III radioimmunoassay, an increase in the level of synthesis was found to occur before previtellogenesis (0–24 h). A second increase appeared at the onset of vitellogenesis (72–83 h) and continued until the end of vitellogenesis (96 h) and the occurrence of chorionation (120 h). Since sexual receptivity develops with vitellogenesis, the significantly higher levels of JH III biosynthesis in vitro at this time supports a possible role for JH in the acquisitive of receptivity.
Key words. Juvenile hormone III biosynthesis; radioimmunoassay; ovarian maturation; sexual receptivity; *Calliphora vomitoria*.

In higher flies, vitellogenesis and oocyte growth both appear to be controlled by juvenile hormone (JH) and ecdysteroids[1,2]. In *Calliphora vomitoria*, parallel fluctuations occur in the ecdysteroid titers in the hemolymph and ovaries during oocyte growth, with a simultaneous drop during chorionation[3]. In this same species, destruction of the corpora allata (CA) before oocyte growth begins prevents ovarian development and sexual receptivity. Both processes are restored by the application of a JH analogue, S-methoprene, to the allatectomized females[4]. These same results have been obtained with *Musca domestica*[5], *Drosophila melanogaster*[6], and *Lucilia cuprina*[7], thus suggesting that JH plays a major role in ovarian development and receptivity in flies.
Knowledge of the JH titers in adult females is critical to an understanding of the function of JH in these processes. However, little information is available on the identification and titers of the JHs in Diptera, except for *Aedes aegypti*[8] and *Drosophila hydei*[9–11], in which only JH III is present in whole body extracts of larvae and adults.
We confirmed by gas-liquid chromatography and mass spectrometry (GLC-MS) that JH III is the only JH in *C. vomitoria*. They also document that specific fluctuations in JH biosynthesis in vitro by CA taken during the first gonadotropic cycle correlate with the development of female receptivity, indicating that JH titers may fluctuate in the same way.

Materials and methods. Adults of *C. vomitoria* were reared at 25 °C under a L:D 12:12 photoperiod and were fed daily with a diet of water, sugar and minced meat. Under these conditions, the gonadotropic cycle lasts 5 days. Experimental animals were isolated at emergence. Behavioral observations were carried out at a time of day determined to be optimal for sexual activity, between 09.00 and 11.00 h. Females of known imaginal age (3, 6, 12, 24, 48, 72, 83, 92, 96 or 120 h) were kept together with 5-day-old mature and sexually motivated males for a period up to 1 h. Female receptivity was scored by the claspering of male and female genitalia, stopped after 4 min to prevent insemination. Ovarian development in both unreceptive and receptive females was evaluated by the stage of oocyte development based on the observation that the polytrophic ovary contains 1, 2, 3 and 4 oocytes in succession and by the degree of completion of vitellogenesis in the terminal follicle. Vitellogenesis began at 72 h and finished at 96 h. Because of the rapidity of this phenomenon, JH production levels were measured at 83 h and 92 h. For JH identification, ten CA taken from females 48 h after emergence and thus well before the onset of vitellogenesis (72 h) were incubated in triplicate for 4 h at 25 °C in 100 µl of medium TC 199 (with Hanks' salts, L-glutamine, and 25 mM HEPES buffer – Gibco). Non-polar products of the glands were extracted with 1 ml of pentane. The efficiency of this