A Serum-Free *in vitro* Culture System for Crayfish Organs

Gerd Gellissen, Marco Traub, and Klaus-Dieter Spindler

Institut für Zoologie III, Universitätsstraße 1, D-4000 Düsseldorf 1, Bundesrepublik Deutschland

Z. Naturforsch. **41c,** 472–476 (1986); received December 16, 1985

Crayfishes, Protein Biosynthesis and Secretion, Hypodermis, Midgut Gland, Hemocyanin

Midgut gland and hypodermis of the crayfish *Astacus leptodactylus* have been cultured in a serum-free medium for several days. The medium consists of 1 part of van Harreveld solution and 1 part of an amino acid mixture supplemented with 1.2 mM Na_2HPO_4, 12 mM Hepes and 80 mM glucose. The antibiotics penicillin (15 mg/l) and streptomycin (25 mg/l) were added for long term culturing. This medium, called TG medium, allows the maintainance of the tissues for more than 100 h without any loss of their viability with respect to protein synthesis and secretion.

Introduction

The compelling interest in regulation of arthropod development by molting hormones has been greatly stimulated by different *in vitro* systems, which allow to examine the action of the hormones on isolated organs and tissues [1, 2]. One of the key events are the hormone-induced alterations of gene expression leading to a changed pattern of polypeptide biosynthesis in target tissues [3]. A prerequisit to study these phenomena in isolated tissues is the establishment of a medium, which enables a reliable and reproducible analysis of protein biosynthesis and secretion after several days of culturing.

For *A. leptodactylus* a culture system has already been described [4]. Being a useful tool for quantitative studies on protein and nucleic acid biosynthesis as well as for description of uptake and metabolism of ecdysteroids [4–7] we found that qualitative studies of protein biosynthesis and secretion are impaired by the presence of peptides and amino acids originating from a horse serum supplement in the system. We felt obliged to establish a more defined medium in which supplementation with horse serum is avoided. As model tissues we analysed the midgut gland and the hypodermis characterized as target tissues for ecdysteroids [8] and revealing characteristic changes of protein production in correlation to the molting cycle [9].

Reprint requests to Prof. Dr. K.-D. Spindler.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0400–0472 $ 01.30/0

Materials and Methods

Animals

The crayfish *A. leptodactylus* was obtained from a local supplier. Rearing and molting stage determination are as described [10, 11]. Only male animals from the intermolt stage C4 were used for the experiments.

Tissue dissection and culturing

Before dissection the crayfishes were surface-sterilized with 70% ethanol. The dissected tissues were washed three times in sterile van Harreveld saline [VHS] [12] and transferred into sterile szintillation vials. TG medium (see Table I), sterilized by filtration through a membrane (Sartorius 0.2 µm) was added as 2 ml. Incubation was performed at 18 °C under constant gentle shaking on a horizontal shaker (IKA Combimag). During long-term culturing tissues were washed after every 24 h and transferred into new vials containing fresh medium.

Protein production by crayfish tissues in vitro

Freshly dissected or long-term cultured tissues were washed as above and transferred into sterile vials containing 2 ml of incorporation medium. The incorporation medium is identical with TG medium (see Table I) except that the cold methionine is replaced by 70 µCi [^{35}S]methionine (NEN, 600 Ci/mM). Incorporation was performed by incubation as above for appropriate times. After incorporation of [^{35}S]methionine newly synthesized polypeptides in the medium were precipitated from 20 µl aliquots with 10% TCA. The precipitates were sedimented by centrifugation and washed five times with 5% TCA.

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung") beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

The final sediments were dissolved in 1 N NaOH and incorporation of [^{35}S]methionine was analysed by liquid szintillation counting in a Packard Tricarb 460C counter.

For analysis of incorporation into the intracellular protein fractions, the tissues were washed three times in 50 ml van Harreveld saline and then homogenized with an Ultraturrax in 2 ml of a solution containing 1 part van Harreveld saline and 1 part 2 M NaCl in 250 mM EDTA, pH 7.5. The homogenate was centrifuged and from a 13,000 × *g* supernatant (operationally called "cytosol") newly synthesized proteins were precipitated from 20 µl aliquots and analysed as described for the secreted proteins.

SDS polyacrylamide gel electrophoresis and fluorography

For qualitative analysis TCA sediments, obtained as described above, were separated by SDS-PAGE according to Lämmli [13] on 10–15% linear gradient gels and stained with Coomassie-Blue. For analysis of labelled polypeptides the stained gels were incubated for 20 min in a fluorographic reagent solution (Amplify, Amersham), dried on Whatman 3M paper and exposed to X-ray films (Kodak-Xomat) for appropriate times at −70 °C.

Quantification of proteins

Proteins were quantified according to Bradford [14] using BSA as standard.

Results

In our attempt to establish a serum-free medium for crayfish organs we tested supplementation with different concentrations of amino acids and minerals. As an initial criterium for survival we examined the tissues by staining with 0.1% Trypan Blue and microscopy. In our tests we mixed one part van Harreveld saline with one part of an amino acid solution from an insect medium [15], which had been used for a similar type of study [16, 17]. The best results were obtained with concentrations as listed in Table I being in a range comparable to the concentrations of another serum-free crustacean medium [18]. Survival was further improved by supplementation with 1.2 mM Na_2HCO_3, 12 mM HEPES and 80 mM glucose. Thus the sugar concentration (14.5 g/l) was lowered to a more physiological range (Astacus serum: about 9 g/l) as compared to the first serum-containing medium [4].

For long-term culturing we added the antibiotics penicillin and streptomycin as 15 mg/l and 25 mg/l respectively. Further protection against contamination was achieved by washing with van Harreveld saline and transfer into fresh medium after every 24 h of culturing; the temperature for incubation was lowered from 25 °C in former studies to 18 °C.

Under these conditions the tissues survived for more than 100 h. The viability of long-term cultured tissues was proved by morphological and physiological comparison with freshly prepared parts of the tissues. A few electron microscopical studies of the hypodermis revealed the integrity of organelles and

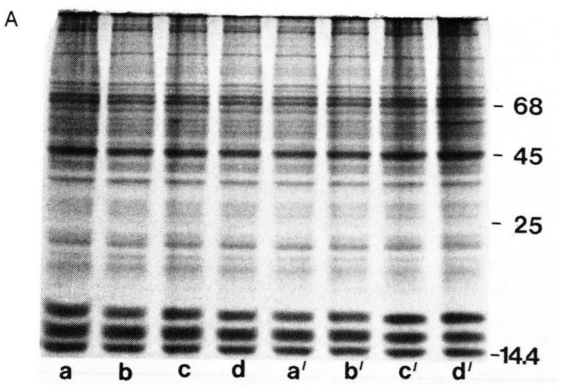
Table I. Composition of the TG-medium (final concentrations).

	mM	g/l
Half-concentrated van Harreveld-saline		
NaCl	102.6	5.992
$CaCl_2$	7.0	0.770
KCl	2.7	0.201
$MgCl_2$	1.3	0.264
$NaHCO_3$	1.2	0.101
Amino acids		
ALA	0.167	0.015
ARG	0.167	0.035
ASN	0.167	0.025
ASP	0.167	0.022
CYS	0.067	0.012
GLN	0.167	0.025
GLU	0.167	0.024
GLY	0.333	0.025
HIS	0.500	0.078
ISO	0.067	0.009
MET	0.067	0.010
PHE	0.067	0.011
PRO	0.167	0.019
SER	0.333	0.035
THR	0.067	0.008
TRP	0.067	0.014
TYR	0.033	0.006
VAL	0.067	0.008
LYS	0.167	0.031
LEU	0.067	0.009
Other components		
Na_2HPO_4	1.2	0.012
Hepes	12.0	2.750
Glucose	80.0	14.500
Penicillin		0.015
Streptomycin		0.025

pH: 7.8
Osmolarity: 300 mOsmol

confirmed the results of Trypan Blue uptake experiments. In our physiological experiments we focussed our comparison on the analysis of polypeptides, a tool for our anticipated project to examine the function of ecdysteroids *in vitro*.

The polypeptide pattern of cytosolic proteins remained constant in a reproducible fashion in hypodermis as well as in the midgut gland (Fig. 1). The



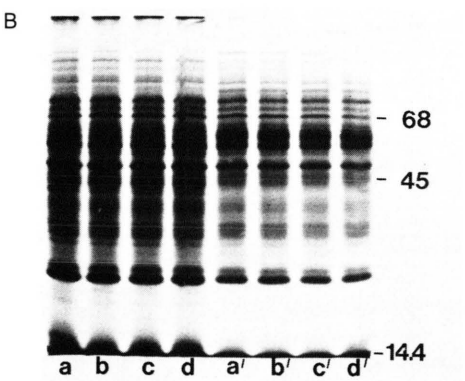


Fig. 1. Comparison of the polypeptide pattern of the cytosol of freshly dissected and long-term cultured tissues. Tissues were split into two parts. One half was cultured for 75 h. TCA precipitates from the cytosolic fraction of both parts were separated by SDS-PAGE on 10–15% linear gradients. Staining with Coomassie Blue. M_r standardization is indicated as determined by comparison with a protein standard mixture.

A: polypeptide patterns of the hypodermis.
B. polypeptide pattern of the midgut gland.
a–d: freshly prepared parts of the tissue of different animals.
a'–d': corresponding parts after 3 days of culturing.

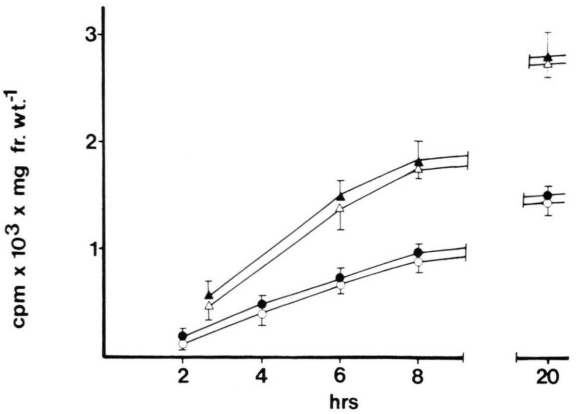


Fig. 2. Secretion of newly synthesized polypeptides into the medium. Comparison of secretion kinetics between freshly prepared and long-term cultured tissues. Tissues were split as above. One part was incubated immediately into 2 ml of incorporation medium containing 70 μCi [^{35}S]methionine, the other part was cultured for 3 days in TG-medium prior to the transfer into the incorporation medium. 20 μl aliquots of the medium were analyzed for labelled polypeptides as described in Material and Methods and the time indicated. ($\times \pm$ S.D.; $n = 4$) hypodermis freshly dissected (△) or after 3 days of culturing (▲) and midgut gland freshly dissected (○) or after 3 days of culturing (●).

rates of total protein output are identical for freshly prepared and long-term cultured tissues (Fig. 2). Tissue-specific differences became apparent when the pattern of newly synthesized proteins was analysed by SDS-PAGE and subsequent fluorography. Whereas in the hypodermis the pattern of biosynthesis remains unchanged after long-term culturing (Fig. 3A) the midgut gland switched to an altered pattern of biosynthesis. Despite of an unchanged pattern in cytosolic polypeptides after staining with Coomassie Blue (see Fig. 1), incorporation of [^{35}S]methionine into different protein fractions was observed. In freshly prepared tissues mainly polypeptides of some 80 kDa are produced, subunits of the major serum protein, the hemocyanin, as shown by immunological procedures (data not shown). In long-term cultured midgut gland, hemocyanin production is turned off leading to an enhanced incorporation of [^{35}S]methionine into other polypeptide fractions. The altered pattern of biosynthesis is reproducible and does not result from degradation of labelled hemocyanin polypeptides.

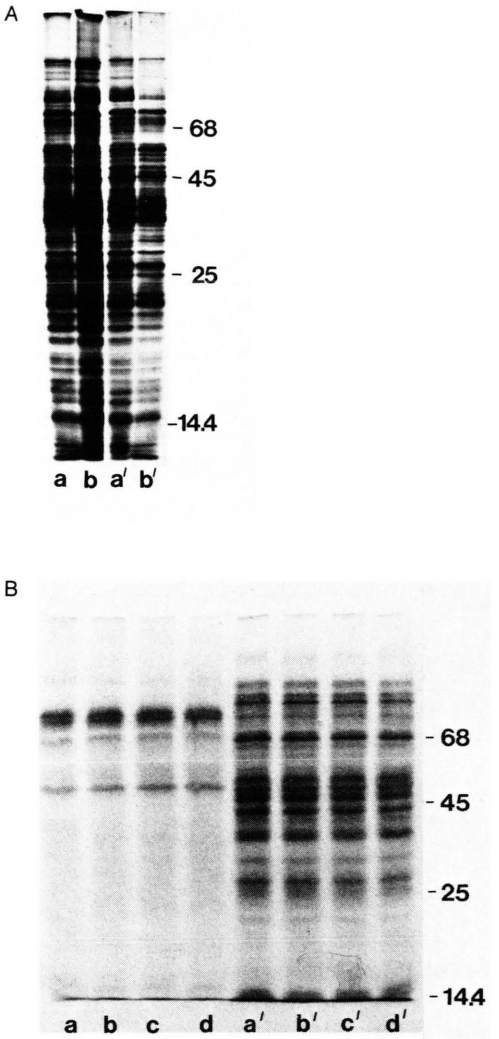


Fig. 3. Biosynthesis of polypeptides of freshly prepared and long-term cultured tissues. Tissues were incubated and prepared as in Fig. 2. Labelled polypeptides were precipitated from the cytosolic fraction and analyzed by SDS-PAGE as in Fig. 1 and subsequent fluorography. M_r standardization is indicated as determined by comparison with protein standard mixture.
A: hypodermis.
B: midgut gland.
a–d: freshly prepared tissues.
a′–d′: corresponding biosynthesis pattern after 3 days of culturing.

Discussion

Many studies on protein biosynthesis of invertebrates have been made using tissue culturing as a tool [1, 2, 19, 20]. Such studies are more or less linked to insect systems. In crustaceans, tissue culture is described for a limited number of species and organs [4, 18, 21–26] and in most cases short-term culturing in serum-containing media has been applied. Moreover, detailed studies on polypeptides from the hypodermis and the midgut gland by SDS-PAGE and fluorographic methods do not exist in the crustacean literature.

In insects, different classes of plasma proteins have been described, which are produced by the fat body [16, 27, 28]. From our present study it can be concluded that the analoguous organ, the midgut gland, may have a similar function in synthesizing plasma proteins in crustaceans. The major hemolymph protein, the hemocyanin, is produced by this organ. At present we are investigating whether this protein is exclusively made by the midgut gland. In spiders the hemocyanin is known to be produced by the pericard [29]. In our present study we observed that hemocyanin production is turned off after long-term culturing. Though we do not know the reason for this phenomenon, we believe that our medium has to be supplemented with additional factor(s), specially required for hemocyanin biosynthesis, such as copper. We are planning to investigate this problem in more detail, because nothing is known about special requirements or feed back mechanism in its biosynthesis. Despite this difference in the midgut gland we believe that our newly composed medium fulfills all criteria for an optimized further analysis of polypeptide production after long-term culturing. It has already been applied to study the ecdysteroid-induced alterations in hypodermis [30].

Acknowledgement

We gratefully acknowledge the financial support of this work by the Deutsche Forschungsgemeinschaft.

[1] J. Koolman and K.-D. Spindler, Endocrinology of Insects (R. G. H. Downer and H. Laufer, eds.), p. 179, Alan Riss Inc., New York 1983.
[2] E. Kurstak, K. Maramorosch, and D. Dübendorfer, Invertebrate Systems in Vitro, Elsevier, Amsterdam 1980.
[3] P. Karlson, Hoppe-Seyler's Z. Physiol. Chemie **364,** 1067 (1983).
[4] K. Daig and K.-D. Spindler, Z. Naturforsch. **34c,** 1243 (1979).
[5] P. Kuppert, K. Daig, M. Londershausen, and K.-D. Spindler, Regulation of Insect Development and Behaviour (F. Sehnals, A. Zabzà, J. J. Menn, and B. Cymborowsky, eds.), p. 663, Technical University of Warsaw 1981.
[6] K. Daig and K.-D. Spindler, Mol. Cell. Endocrinol. **31,** 93 (1983).
[7] K. Daig and K.-D. Spindler, Mol. Cell. Endocrinol. **32,** 73 (1983).
[8] K.-D. Spindler, L. Dinan, and M. Londershausen, Biosynthesis and Mode of Action of Invertebrate Hormones (J. Hoffmann and M. Porchet, eds.), p. 255, Springer, Heidelberg 1984.
[9] M. Bielefeld, G. Gellissen, and K.-D. Spindler, Insect Biochem. **16,** 175 (1986).
[10] F. van Herp and C. Bellon-Humbert, Aquaculture **14,** 289 (1978).
[11] P. Kuppert, S. Wilhelm, and K.-D. Spindler, J. Comp. Physiol. **128,** 95 (1978).
[12] A. van Harreveld, Proc. Exp. Biol. **34,** 428 (1936).
[13] U. Lämmli, Nature **227,** 680 (1970).
[14] M. Bradford, Anal. Biochem. **77,** 428 (1976).
[15] G. R. Wyatt, T. T. Chen, and P. Couble, Invertebrate Tissue Culture, Applications in Medicine, Biology and Agriculture (E. Kurstak and K. Maramorosch, eds.), p. 195, Academic Press, New York 1976.
[16] G. Gellissen and G. R. Wyatt, Can. J. Biochem. **59,** 648 (1981).
[17] G. Gellissen and G. R. Wyatt, unpublished results.
[18] E. M. Wolin, H. Laufer, and D. F. Albertini, Dev. Biol. **35,** 160 (1973).
[19] C. Vago, Invertebrate Tissue Culture, Academic Press, New York 1971.
[20] E. Kurstak and K. Maramorosch, Invertebrate Tissue Culture, Applications in Medicine, Biology and Agriculture, Academic Press, New York 1976.
[21] J. Berreur-Bonnenfant, Invertebrate Tissue Culture, **Vol. II** (C. Vago, ed.), p. 181, Academic Press, New York 1972.
[22] S. N. Oyama and F. I. Kamemoto, Crustaceana **18,** 309 (1970).
[23] U. E. H. Fyhn and J. D. Costlow, Biol. Bull. **149,** 316 (1975).
[24] M. R. Lewis, Anat. Record. **10,** 287 (1976).
[25] C. H. Holland and D. M. Skinner, Biol. Bull. **150,** 222 (1976).
[26] E. S. Chang and J. D. O'Connor, Proc. Natl. Acad. Sci. **74,** 615 (1977).
[27] G. R. Wyatt and M. L. Pan, Annu. Rev. Biochem. **47,** 779 (1978).
[28] F. Engelmann, Adv. Insect Physiol. **14,** 49 (1979).
[29] B. Kempter, Naturwissenschaften **70,** 255 (1983).
[30] M. Traub, G. Gellissen, and K.-D. Spindler, submitted.