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Astakine1 forms protein complex in plasma

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ABSTRACT

Astakine 1 is a small cytokine-like peptide which is directly involved in hematopoiesis in crustaceans. Astakines are present in many different invertebrate groups primarily in arthropods. In this study we found that astakine1 was present as a high molecular weight (HMW) complex in plasma. It is known that calcium concentration are fluctuating in several crustaceans especially during the molting process. This HMW-complex was formed under low calcium concentrations in plasma and could be partially reversed provided calcium was added. The biological function of the naïve astakine1 and that in the HMW complex was about the same, but if the protein is to be isolated or studied for its function it is important to know about this property of astakine1 which may previously have hampered isolation and functional studies in other animals than freshwater crayfish.

1. Introduction

Astakine1 (Ast1) is a hematopoietic cytokine in crustaceans, which is a crucial factor in proliferation and differentiation of hematopoietic stem cells (HPT) [1]. It is controlling progenitor cell proliferation by regulating the ECM structure, extracellular transglutaminase (TGase) activity and clotting protein (CP), which results in inducing HPT differentiation along the semi-granular lineage [2–4].

Ast1 is a small molecule of 82 amino acids with a calculated molecular weight of approximately 8.7 kDa and a prokineticin (PROK) domain constitutes the main part of the mature protein [5]. In vertebrates, PROK-containing proteins are involved in a variety of biological processes such as hematopoiesis, angiogenesis, neurogenesis and circadian rhythms [6,7]. All vertebrate PROKs contain the amino acids AVIT at the N-terminal, but this conserved sequence is not present in the crayfish Ast1, or in other known invertebrate astakines [5,8]. We have purified and characterized Ast1 from crayfish hemolymph [5], but so far no other studies using native Ast1 have been published. This may be due to that Ast1 is unstable during purification and is present in low quantity in plasma. The difficulties to obtain large amount of purified native Ast1 makes detailed studies of its molecular interactions, for example with receptor proteins challenging. The aim of this study is to understand the formation of high molecular weight astakine1 (HMW-Ast1) upon different physiological conditions in crayfish plasma. In this study we report that the small cytokine-like Ast1 peptide can form a HMW complex under low calcium concentrations in plasma and that both this high molecular weight form and the low molecular weight form of astakine1 are equally functional.

2. Materials and methods

2.1. Experimental animals

Freshwater crayfish, *Pacifastacus leniusculus* were obtained from Lake Erken, Sweden. The animals were kept in aerated tap water at 10 °C. Only healthy intermolt animals were used.

2.2. Collection and preparation of cell-free plasma

The hemolymph from individual crayfish was collected in an ice-cold anti-coagulant buffer, (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) at a dilution 1:1 hemolymph:anticoagulant [9]. The hemocytes were removed by centrifugation at 800 × *g* for 5 min at 4 °C. The plasma supernatant served as cell-free plasma. Then, 500 µl of cell-free plasma was subjected to ultracentrifugation 200,000 × *g* for 1.5 h at 4 °C. The resulting supernatant is named as plasma-UC and is a crude source of Ast1.

Hemolymph collected and mixed at a ratio of 1:1 vol with anticoagulant was analyzed for levels of Ast1 by western blotting. Further, the presence of Ast1 in plasma-UC after addition of EGTA (final concentration at 5 mM), or $CaCl_2$ was examined by Western blot analysis. A total amount of 10 µg protein was then precipitated with acetone for

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12 h at −20 °C, and then subjected to Western blot analysis for determination of Ast1, using a polyclonal anti-Ast1 antibody [5].

2.3. Western blot

Plasma-UC preparations were separated by 12.5% SDS-PAGE in protein loading buffer (Laemmli sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% (v/v) glycerol, 0.1 M DTT, 0.01% bromophenol blue, pH 6.8) [10] and then electro-transferred to a polyvinylidene fluoride membrane (PVDF, GE Healthcare) for 2 h. The blot was blocked by immersion in 10% skimmed milk solution in PBS-T (0.5% tween-20 in 1X PBS buffer, pH 7.4) for 1 h at room temperature. The membrane was then incubated with a specific antibody to Ast1 at a dilution of 1:5000 and anti-beta-1,3-glucan-binding protein antibodies (βGBP) [11] at a dilution of 1:20,000 was used as control. Anti-rabbit antibody conjugated with horseradish peroxidase (GE Healthcare) at a 1:7500 dilution was used as the secondary antibody. For detection, the ECL Western blotting reagent kit (BioRad) was used according to the manufacturer's instructions.

2.4. Circulating hemocyte count

To investigate total hemocyte count (THC), protein concentration and Ast1 concentration the samples were collected without anti-coagulant. Hemolymph was withdrawn from the abdominal hemocoel by using a needle (18G) into a 2 ml sterile syringe on ice and 50 µl of hemolymph was collected and immediately fixed in 10% formalin solution for determining total hemocyte count (THC). Hemolymph was collected every second week from 5 crayfish that were maintained in aquaria during a year.

2.5. Detection of calcium concentration in cell-free plasma

To determine the calcium level in cell-free plasma, a calcium colorimetric method (MAK022, Sigma) was used according to the manufacturer's instructions. Briefly, 25 µL of cell-free plasma was added to the reaction to obtain a total volume of 100 µL per reaction, which contained 45 µL of chromogenic reagent and 30 µL of calcium assay buffer. After incubation the mixture at room temperature for ca 5 min, the chromogenic complex formed between calcium ions and o-cresolphthalein was measured at 575 nm. The concentration of calcium ions is calculated relative to a standard curve.

2.6. Hemocyte culture

The hemolymph from individual crayfish was collected in anti-coagulant and diluted 1:1. After centrifugation (800 × *g* for 5 min at 4 °C) to collect the hemocyte pellet, the cells were re-suspended in 1 mL of 0.15 M NaCl. The hemocytes were cultured in 96-well plate at a density of 5×10^4 cells/well in 0.15 M NaCl and at 16 °C for 24 h. Then, 300 µl of cell free "hemocyte-conditioned" NaCl and hemocytes were collected separately, and subjected to acetone precipitation at −20 °C. To examine the effect of calcium or DTT on Ast1, the "hemocyte-conditioned" NaCl was treated with 5 or 50 mM $CaCl_2$ or 0.4 M DTT at RT for 1 h before acetone precipitation. The protein pellet was collected by centrifugation at 13,000 × *g* for 20 min at 4 °C. After drying at RT for 10 min, the protein pellet was dissolved in protein loading buffer and subjected to Western blot assays.

2.7. Statistical analysis

The total protein concentration and total hemocyte count during a year in Fig. 6 are shown as mean ± standard deviation (SD) value, and the statistical analysis was performed using the one-way ANOVA followed by Tukey's test. For comparisons the protein concentration, total calcium level and total hemocyte count between two groups of plasma in Figs. 8 and 9, *t*-test was performed. Significant differences were considered at $P < 0.05$. Each symbol represents individual crayfish and the results are reported as mean ± SD value.

3. Results and discussion

3.1. Astakine 1 structure is affected by calcium chelating agents

When we collected hemolymph from crayfish in an anti-coagulant containing EDTA at low pH [9], we noticed that the size of Ast1 as analyzed by western blotting was around 65–70 kDa (Fig. 1). We named this high molecular form of Ast1 as HMW-Ast1. We then addressed the question of whether the presence of HMW-Ast1 was due to the low pH or EDTA or both in combination. Thus, we used a plasma preparation after ultracentrifugation in which most of the hemocyanin was removed, and this preparation contained only Ast1 at low molecular weight (LMW-Ast1). Then, ultra-centrifuged plasma (plasma-UC) was treated with EGTA at two different concentrations (0.5 or 5 mM) at pH 4.6 and at neutral pH. As shown in Fig. 1, HMW-Ast1 was detected after treatment with EGTA at 5 mM regardless of pH (4.6 or 7.0) and at 0.5 mM bands at both low and high molecular weight were detected (Fig. 1). As observed in Fig. 1, HMW-Ast1 is detected at both low (pH 4.6) and neutral pH (pH 7.0). This result indicates that a low pH did not affect the formation of HMW-Ast1. However, when plasma was treated with EGTA (0.5 or 5 mM), there was a change on LMW-Ast1, indicating that the presence of Ca^{2+} (or other divalent ions) may be important to keep Ast1 in a LMW-Ast1 form.

Therefore, we tested the effect of calcium on HMW-Ast1, by adding calcium to plasma-UC containing only HMW-Ast1 that was prepared without anti-coagulant. However, this treatment did not affect the levels of HMW-Ast1 (Fig. 2), and thus once HMW-Ast1 is formed in plasma it seems to be a stable complex. We also treated HMW-Ast1 plasma with DTT but no change in molecular weight occurred (Fig. 4). Ast1 has a calculated mass of 8.7 kDa and pI of 5.0. This means that at normal pH in crayfish hemolymph (about 6.8) the protein is negatively charged and possibly can bind divalent cations like Ca^{2+}. Ast2 [13] on the other hand has a calculated pI of 6.8, and we have previously shown that recombinant Ast2 moves as a monomer in SDS-PAGE under non-reducing conditions, while recombinant Ast1 form multimers with an apparent molecular weight of about 370 kDa [13]. Since DTT did not change the HWM-Ast1, we concluded that this complex was not only a result of disulfide bonds between Ast1 monomers in the presence of EGTA or anti-coagulant. These structural changes in Ast1 in different solutions may explain the difficulties to purify this protein from plasma [5].

3.2. Astakine 1 is released from hemocytes as HMW-Ast1

Since crayfish hemocytes are known to contain Ast1 and that this Ast1 is secreted into plasma [5], we next tested the presence of HMW-Ast1 after hemocyte secretion *in vitro*. When hemocytes were cultured in 0.15 M NaCl for 24 h at 16 °C, HMW-Ast1 was found in the surrounding ("hemocyte-conditioned") NaCl (Fig. 3A) while only LMW-Ast1 was detected in the hemocyte lysate (Fig. 3B). In contrast, when HPT cells were incubated in L-15 medium only LMW-Ast1 could be detected both in conditioned medium and in a HPT lysate (data not shown). These results indicate that HMW-Ast1 is formed after secretion from the hemocytes into the external milieu, and that other proteins released from the hemocytes are involved in this complex formation, while HPT cells do not secrete such proteins.

Next we decided to observe the effect of calcium on the formation of HMW-Ast1 in hemocytes cultured in 0.15 M NaCl. After collecting the hemocyte-conditioned NaCl at 24 h, $CaCl_2$ was added to the solution and incubated for 1 h at RT before it was subjected to Western blot analysis. When $CaCl_2$ was added to a final concentration of 5 mM, LMW-Ast1 was formed (Fig. 4), indicating that Ca^{2+} may dissolve the

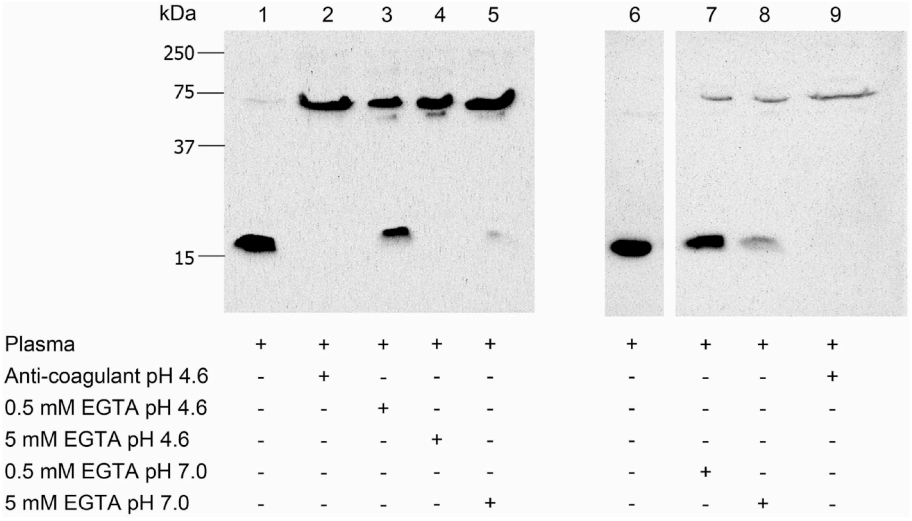

Fig. 1. Effect of EGTA or anti-coagulant on the apparent molecular weight of Ast1. Plasma-UC was treated with anti-coagulant or EGTA at different concentrations and different pH values and then subjected to western blotting using anti-Ast1 antibody at a dilution of 1:5000. Plasma-UCs were prepared from crayfish number 1 (lane 1–5) and crayfish number 2 (lane 6–9). Lanes 1 and 6: plasma-UC; lanes 2 and 9: plasma-UC diluted 1:1 with anti-coagulant; lane 3: plasma-UC treated with 0.5 mM EGTA at pH 4.6; lane 4: plasma-UC treated with 5 mM EGTA at pH 4.6; lanes 5 and 8: plasma-UC treated with EGTA 5 mM at pH 7.0 and lane 7: plasma-UC treated with 0.5 mM EGTA at pH 7.0. The amount of protein in all lanes are 10 µg. This experiment was repeated four times with similar results.

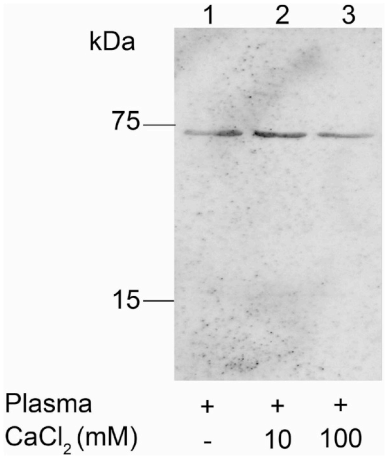

Fig. 2. The effect of calcium addition to plasma containing HMW-Ast1. Plasmas were collected without anti-coagulant. After ultra-centrifugation the resulting plasma-UC was treated with calcium at different concentrations and then analyzed by western blotting. Lane 1: plasma-UC; lane 2: plasma-UC treated with 10 mM $CaCl_2$; lane 3: plasma-UC treated with 100 mM $CaCl_2$. The amount of protein in all lanes are 10 µg. This experiment was repeated four times with plasma-UC from different animals.

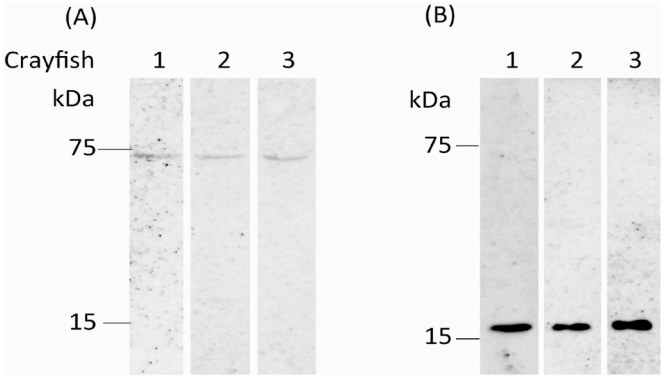

Fig. 3. The presence of HMW-Ast1 in hemocyte-conditioned NaCl (A) or a hemocyte lysate (B). Hemocytes from different crayfish individuals (crayfish 1–3), were cultured in 0.15 M NaCl at a density of 50,000 cells/well, then the hemocyte-free NaCl and hemocyte lysate were collected separately after 24 h culture, and analyzed by western blotting using an Ast1 antibody. This experiment was repeated six times with similar results.

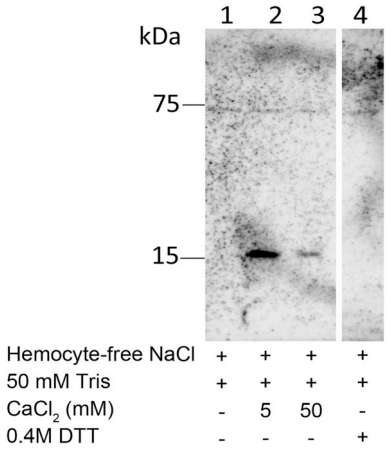

Fig. 4. The effect of calcium on HMW-Ast1 in the hemocyte-conditioned NaCl. Hemocytes were cultured in 0.15 M NaCl at a density of 50,000 cells/well, then the hemocyte-free NaCl was collected after 24 h incubation at 16 °C, and 300 µl was treated with $CaCl_2$ at 5 or 50 mM, or 0.4 M DTT for 1 h before acetone precipitate and then subjected to western blotting. Lane 1: hemocyte-conditioned NaCl after 24 h incubation at 16 °C; lane 2: hemocyte-conditioned NaCl treated with 5 mM $CaCl_2$; lane 3: hemocyte-conditioned NaCl treated with 50 mM $CaCl_2$ and lane 4: hemocyte-conditioned NaCl treated with 0.4 M DTT. This experiment was repeated four times with similar results.

HMW-Ast1 complex when this complex was formed in a simple saline solution, while in plasma this was not possible (see above). However, at high $CaCl_2$ (50 mM) the amount of LMW-Ast1 was lower. Moreover, DTT treatment did not affect the HMW-Ast1 band and no LMW-Ast1 was detected after DTT addition (Fig. 4). This result again suggests that HMW-Ast1 does not form as a result of disulfide bonds within plasma.

3.3. The presence of HMW-Ast1 correlates with plasma protein concentration

Interestingly, we observed both LMW-Ast1 and HMW-Ast1 in plasma-UC from different crayfish individuals without any addition of EGTA or anti-coagulant (Fig. 5). Plasma from some crayfish had only LMW-Ast1 and some had only HMW-Ast1, and some had both (Fig. 5A and Supplement Fig. S1). As an internal control the plasma protein the beta-1,3-glucan-binding protein (βGBP) was loaded and as shown in Fig. 5B, no complex formation was observed for this protein, although the polyclonal antibody shows some unspecific bands in the plasma from some individuals. The figure also shows that there is variation in

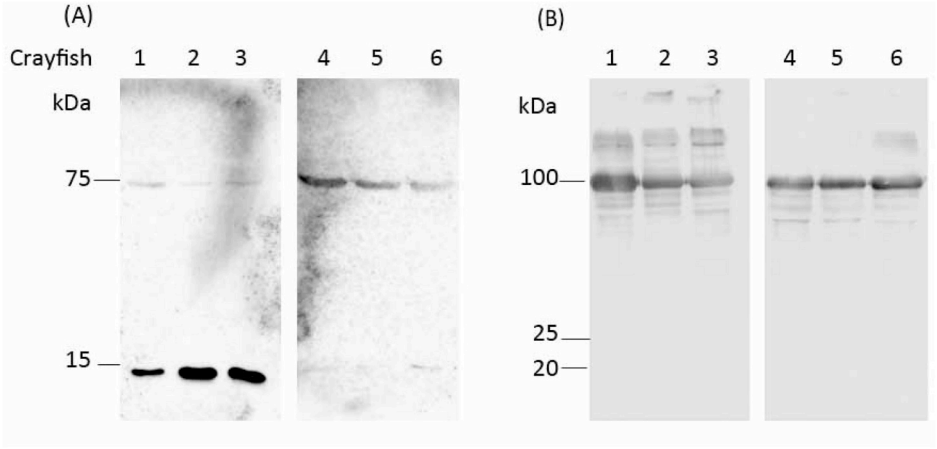

Fig. 5. Detection of LMW-Ast1 and HMW-Ast1 in crayfish plasma. Plasma samples from different crayfish individuals were prepared without anti-coagulant and ultra-centrifuged. Western blots of plasma-UC from different crayfish individuals (1–6) were performed using (A) anti-Ast1 anti-body, or (B) anti-βGBP antibody. The figure shows three representatives of individual crayfish with LMW-Ast1 (crayfish 1–3, lanes 1–3) and with HMW-Ast1 (crayfish 4–6, lanes 4–6). The amount of protein in all lanes is 10 μg.

the amount of plasma proteins, such as βGBP in crayfish (Fig. 5B). This finding suggests that HMW-Ast1 may also occur under natural conditions, regardless of any Ca^{2+} chelating agent. Therefore, we decided to study the presence of LMW-Ast1 and HMW-Ast1 as well as determine the total hemocyte count (THC) once every second week for one year in crayfish that were maintained in aquaria at 10–14 °C. As shown in Fig. 6, THC varied avidly between individuals, but did not show any significant difference between different months. In addition to THC, we also determined the total protein concentration in plasma of these crayfish. Here, the individual variation was very high as well, but there

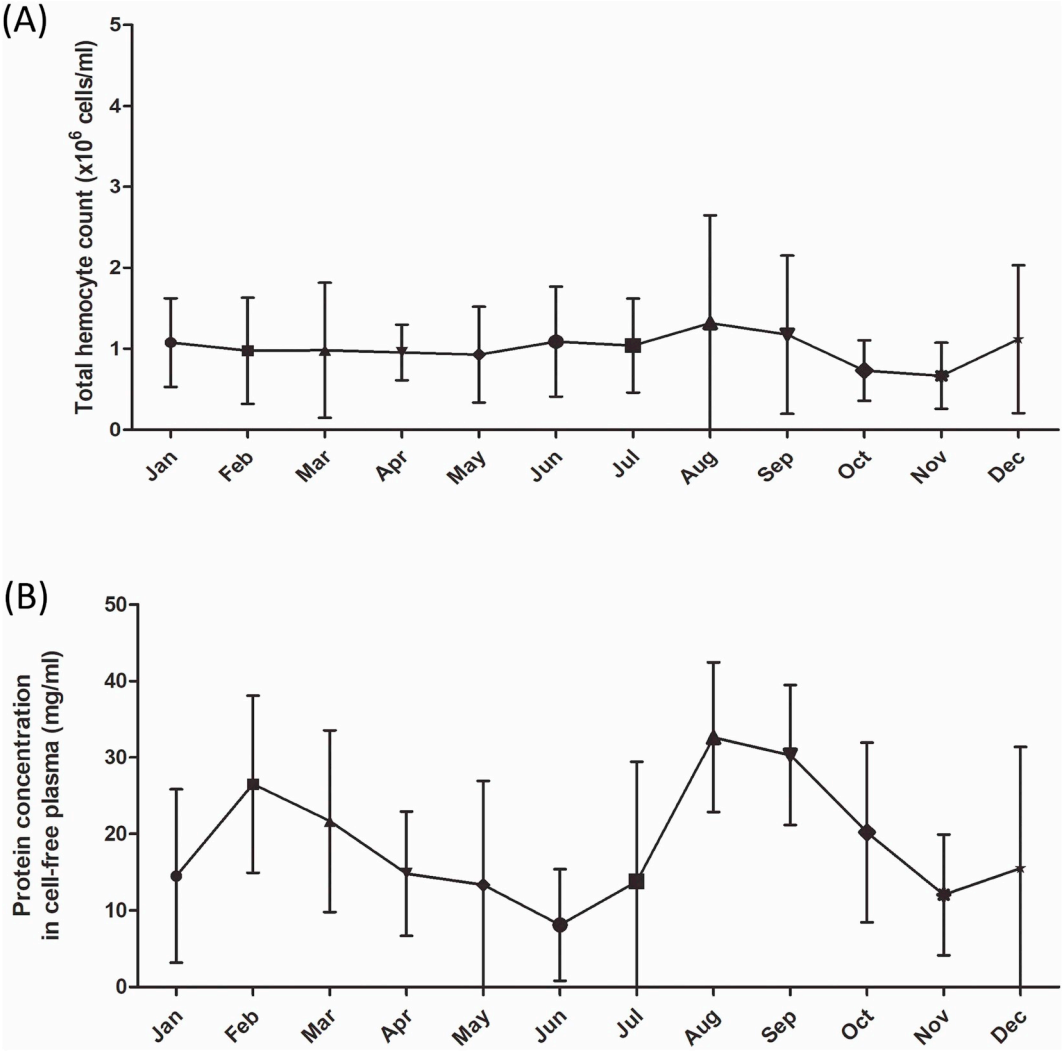

Fig. 6. Analysis of total hemocyte counts (A) and total protein concentration in cell-free plasma (B) in crayfish during one year. Plasma samples were collected without anti-coagulant and hemocytes were removed. Samples from five individuals were collected once every second week from crayfish that were maintained in aquaria during a year. Nine to fifteen samples from different crayfish individuals were determined each month.

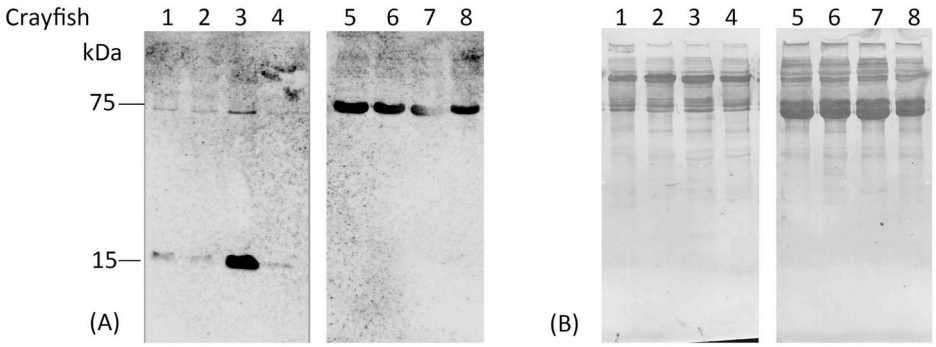

Fig. 7. Comparison of plasma proteins from LMW-Ast1 and HMW-Ast1 in crayfish. Plasma samples were collected without anti-coagulant and plasma-UC from 4 crayfish contained LMW-Ast1 (lanes 1–4) and the other 4 crayfish contained HMW-Ast1 (lanes 5–8). The same amount (10 μg) protein of plasma-UC were precipitated and subjected to 12.5% SDS-PAGE followed by Western blot using anti-Ast1 (A), membrane stained with Coomassie brilliant blue (B).

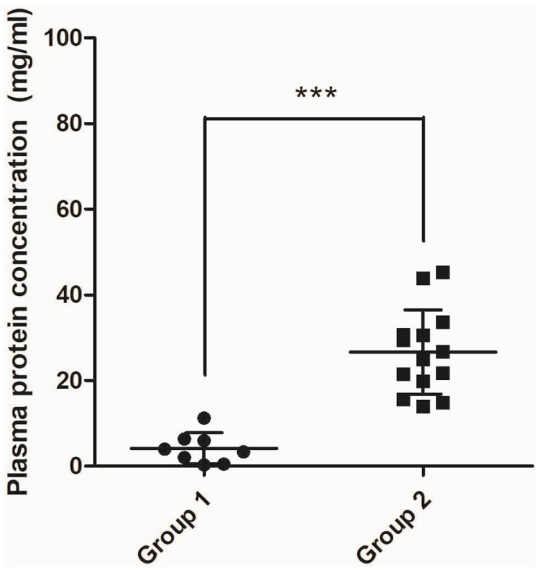

Fig. 8. Protein concentration in plasma characterized by LMW or HMW Ast1 forms. Group 1; LMW-Ast1 Group 2; HMW-Ast1. The data points represent individual crayfish and the error bars represent SD values. Significant differences are indicated by asterisks: ***, $P < 0.001$.

was a tendency towards lower protein concentration around June, which is the molting period, and then a continuous increase in protein concentration from late summer until end of September (Fig. 6B).

Next, we grouped crayfish into two groups according to the presence of LMW-Ast1 and HMW-Ast1, and after western blotting for Ast1 detection we stained the membrane with Coomassie Brilliant Blue in order to look for any possible difference in protein pattern. The result indicates that plasmas with HMW-Ast1 have higher concentrations of protein (Fig. 7). Further, as shown in Table 1, the crayfish could be divided into two groups: (1) LMW-Ast1 with low THC and low plasma protein concentration, and (2) HMW-Ast1 with high THC and high protein concentration, and this result indicates that protein concentration in plasma may affect the formation of HMW-Ast1. Formation of HMW-Ast1 seems to occur in plasma with a high protein concentration, while LMW-Ast1 is present in plasma with low protein concentrations (Fig. 7 and Table 1), and as shown in Fig. 8 a significant difference in protein concentration was observed in plasma from animals with LMW-Ast1 and HMW-Ast1 respectively. However, only a slight difference was found in the number of hemocytes (Fig. 9A) showing no significant effect of THC on the complex formation.

Then, we decided to analyze the amount of total calcium in plasma from crayfish in both groups, using a calcium colorimetric method. The total calcium balance in hemolymph has been reported to be about 8.6 mM in intermolt freshwater crayfish [12], and this is similar to our findings, where the amount of total calcium in plasma varied from 6.5 to 8.9 mM (Fig. 9). However, as shown in Fig. 9B no difference could be

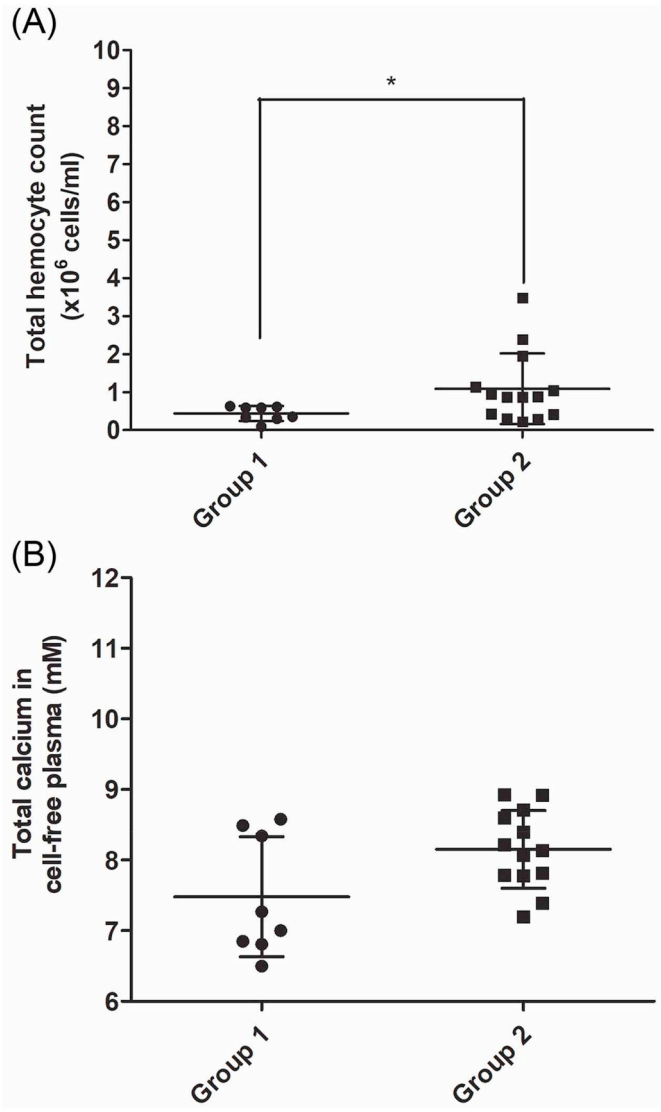

Fig. 9. (A) Total hemocyte count and (B) total calcium in plasma. Group 1; LMW-Ast1 with low protein concentration, Group 2; HMW-Ast1 with high protein concentration. The data points represent individual crayfish and the error bars represent SD values. Significant differences are indicated by asterisks: *, $P < 0.05$.

detected in individuals with HMW-Ast1 or LMW-Ast1.

Since Ast1 is known to induce cell spreading (as well as cell division and differentiation) of HPT cells [5], plasma-UC from group 1 and 2 were added to HPT cell cultures to determine the cell spreading activity. Both plasmas had ability to induce cell spreading with a slightly

Table 1
Data of total hemocyte count and protein concentration were used in this experiment. The groups were divided by the presence of Ast1 forms (This is the data of Figs. 8 and 9).

Group	Sample no.	Total hemocyte count (THC) x10^6 cells/ml	Protein in plasma (mg/ml)
Group 1 LMW-Ast1	1	0.3	0.51
- low THC	2	0.09	6.3
- low protein	3	0.59	11.2
concentration	4	0.59	2.0
	5	0.33	4.0
	6	0.61	3.33
	7	0.35	0.29
	8	0.63	5.96
Group 2 HMW-Ast1	1	1.95	15.6
- high THC	2	1.04	29.48
- high protein	3	1.14	14.0
concentration	4	2.39	30.7
	5	3.48	21.8
	6	0.95	19.8
	7	0.87	21.48
	8	0.43	33.71
	9	0.87	14.83
	10	0.88	30.64
	11	0.29	26.8
	12	0.22	24.9
	13	0.42	43.91
	14	0.3	45.3

higher activity with HWM-Ast1 (Supplementary Fig. S2). Spreading *in vitro* of HPT cells has earlier been shown to be blocked if there is a high extracellular transglutaminase (TGase) activity in the HPT cells [3], and Ast1 functions as an inducer of cell spreading at least partly by inhibiting this TGase activity [4]. Thus, since TGase is dependent on the presence of Ca^{2+} ions, the putative Ca-binding ability of Ast1 at physiological pH as well as the "removal" of active Ast1 by the formation of HMW-Ast1 at low Ca^{2+}, may be a way to regulate TGase activity within the HPT tissue.

4. Conclusion

The molecular conformation of Ast1 *in vivo* seems to depend upon hemolymph protein concentration as well as the level of calcium -chelating agents or calcium concentration, and these circumstances may be reasons for the difficulties to find this small, charged cytokine during separation of crustacean plasma proteins. To our knowledge, our report in 2005 where we first identified Ast1 is so far the only publication about purification of native Ast1 [5] and therefore with this new information it may provide better conditions to isolate native Ast1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.063.

References

[1] I. Söderhäll, Crustacean hematopoiesis, Dev. Comp. Immunol. 58 (2016) 129–141.
[2] K. Junkunlo, K. Söderhäll, I. Söderhäll, Clotting protein - an extracellular matrix (ECM) protein involved in crustacean hematopoiesis, Dev. Comp. Immunol. 78 (2018) 132–140.
[3] X. Lin, K. Söderhäll, I. Söderhäll, Transglutaminase activity in the hema-topoietic tissue of a crustacean, *Pacifastacus leniusculus*, importance in hemocyte ho-meostasis, BMC Immunol. 9 (2008) 58.
[4] R. Sirikharin, K. Junkunlo, K. Söderhäll, I. Söderhäll, Role of astakine1 in regulating transglutaminase activity, Dev. Comp. Immunol. 76 (2017) 77–82.
[5] I. Söderhäll, Y.A. Kim, P. Jiravanichpaisal, S.Y. Lee, K. Söderhäll, An ancient role for a prokineticin domain in invertebrate hematopoiesis, J. Immunol. 174 (2005) 6153–6160.
[6] M.Y. Cheng, C.M. Bullock, C. Li, A.G. Lee, J.C. Bermak, J. Belluzzi, D.R. Weaver, F.M. Leslie, Q.Y. Zhou, Prokineticin 2 transmits the behavioural circa-dian rhythm of the suprachiasmatic nucleus, Nature 417 (2002) 405–410.
[7] J. Monnier, M. Samson, Cytokine properties of prokineticins, FEBS J. 275 (45) (2008) 014-4021.
[8] L. Ericsson, I. Söderhäll, Astakines in arthropods-phylogeny and gene structure, Dev. Comp. Immunol. 81 (2018) 141–151, https://doi.org/10.1016/j.dci.2017.11.005.
[9] K. Söderhäll, V.J. Smith, Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution, Dev. Comp. Immunol. 7 (1983) 229–239.
[10] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[11] B. Duvic, K. Söderhäll, Purification and characterization of a beta-1,3-glucan binding protein from plasma of the crayfish *Pacifastacus leniusculus*, J. Biol. Chem. 265 (1990) 327–332.
[12] M.G. Wheatly, An overview of calcium balance in crustaceans, Physiol. Zool. 69 (1996) 351–382.
[13] X. Lin, M. Novotny, K. Söderhäll, I. Söderhäll, Ancient cytokines, the role of astakines as hematopoietic growth factors, J. Biol. Chem. 285 (2010) 28577–28586.