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Non-specific electrostatic interactions and the conversion of horseshoe crab haemocyanin into a phenoloxidase

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ABSTRACT

Haemocyanin is a haemolymph (blood)-based protein and the functional equivalent to haemoglobin – supplying tissues with oxygen in decapod crustaceans, chelicerates, and shelled molluscs. In addition to oxygen transport, haemocyanin plays several roles in innate immunity, wound healing, and ecdysis. Under certain conditions *in vitro* and *in vivo*, horseshoe crab (*Limulus polyphemus*) haemocyanin is converted into a phenoloxidase-like enzyme, yet the protein-ligand interactions associated with this conversion remain unclear. Negatively charged ligands, such as phosphatidylserine and sodium dodecyl sulphate, represent effective endogenous and exogenous activators, respectively. Herein, we explored the nature of the interaction between haemocyanin and phosphatidylserine. We used several spectroscopic techniques and phenoloxidase assays to follow the electrostatic interactions. Manipulating the ionic strength of the assay resulted in less enzyme activity, and reversed haemocyanin conformational changes associated with phosphatidylserine binding (confirmed by fluorescence emission spectra). The addition of wild type and rearranged peptides – mimicking the P181 to K196 region close to the active site of haemocyanin subunit II – to phenoloxidase assays resulted in less product (dopachrome) formation. We propose that non-specific electrostatic interactions between haemocyanin and endogenous activators such as phosphatidylserine facilitate the switch to a phenoloxidase-like enzyme.

1. Introduction

Like all chelicerates, the Atlantic horseshoe crab *Limulus polyphemus* relies on innate immunity to deter parasites and fight infectious diseases (Coates, 2022). A notable feature of their humoral defences is the inducible phenoloxidase activity of the oxygen transport protein, haemocyanin (Nellaiappan and Sugumaran, 1996; Decker et al., 2001; Baird et al., 2007; Coates and Talbot, 2018; Cunningham et al., 2020; Cubillos et al., 2021). 'True' invertebrate phenoloxidases and their associated activation cascades are considered indispensable for melanin production used in immune reactions and development (Cerenius and Söderhäll, 2021). More recently, Shin et al. (2024) presented evidence for *Drosophila* crystal cells (haemocytes) assisting the tracheal system in modulating haemolymph oxygen tension using intracellular prophenoloxidase 2 (PPO2).

The primary role of invertebrate haemocyanins is to increase the oxygen carrying capacity of the haemolymph, yet these proteins can be converted into phenoloxidase-like enzymes in decapod crustaceans, chelicerates, and certain molluscs (reviewed by Coates and Nairn, 2014). Furthermore, haemocyanins function in hormone transport, bacterial agglutination, moulting, generation of anti-infective peptides, and wound healing (Zhuang et al., 2015; Sanggaard et al., 2016; Coates and Costa-Paiva, 2020). In Pacific whiteleg shrimp (*Penaeus vannamei*), activities of haemocyanin subunits are linked to the maintenance of the hepatopancreatic microbiome (Zheng et al., 2021), peptide release to counteract ammonia toxicity (Zhao et al., 2023), and may modulate glucose metabolism via succinate dehydrogenase (Huang et al., 2024). The ability of certain proteins to participate in unrelated (mechanistically distinct) actions is termed moonlighting (Jeffery, 2014); such proteins are now considered common among animals, plants, and microbes.

In experiments designed to identify natural activators of haemocyanin-derived phenoloxidase in the Japanese horseshoe crab (*Tachypleus tridentatus*), Nagai and Kawabata (2000) and Nagai et al.

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Table 1
Synthetic peptides used in haemocyanin-derived phenoloxidase assays.

Peptide	Abbreviation	Sequence	pI[a]	Mw[a]	Predicted structure[b]
Native sequence (P181 to K196; Hc subunit II)	WT	PSTWNPKYFGKKKDRK	10.30	1980.3	12.5 % extended strand and 87.5 % random coil
Rearranged peptide (Ps fixed in place)	RP	**P**YKWS**P**RGFKNKKDKT	10.30	1980.3	12.5 % extended strand and 87.5 % random coil
Altered peptide 1 (2 Ks replaced with Ts)	AP1	PSTWNPKYFGKTKDRT	10.01	1926.16	12.5 % extended strand and 87.5 % random coil
Altered peptide 2 (2 Ks replaced with Es)	AP2	PSTWNPKYFGKEKDRE	8.83	1982.18	12.5 % extended strand and 87.5 % random coil

[a] Molecular weight and pI were predicted using ExPASY (https://web.expasy.org/compute_pi/).
[b] Peptide structural predictions were retrieved from GOR4 (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_gor4.pl).

(2001) found a clotting enzyme and an amphiphilic antimicrobial peptide (tachyplesin), respectively, which could bind to haemocyanin and induce enzyme activity without proteolytic cleavage. In our studies with *L. polyphemus*, the negatively charged phospholipid phosphatidylserine was identified for further characterisation due to the relative stability of the haemocyanin conformational changes, induced upon phospholipid binding, and the resultant enzyme activity (Coates et al., 2011). Conformational changes mimicked closely those observed in haemocyanin activated via SDS micelles – promoting subtle structural movement near/at the entrance to the active site, thereby permitting substrate (diphenol) access to the copper-bound dioxygen for catalysis (Baird et al., 2007). Prolonged incubation with SDS micelles causes haemocyanin denaturation and loss of enzyme activity, but this was not observed when using phosphatidylserine liposomes (Coates et al., 2011). Structural inspection of haemocyanin subunit II reveals there is a positively charged region (181- PSTWNPKYFGKKKDRK -196) close to the dicopper active site and adjacent to the placeholder residue phenylalanine 49, which may serve as a potential binding site for a negatively charged ligand like phosphatidylserine.

In the present study, we sought to further characterise the nature of the haemocyanin-phospholipid interaction that precedes substrate binding and turnover. To that end, we carried out several experiments to gauge whether non-specific electrostatic forces play a role in this protein conversion and/or if the specific amino acid sequence of the structural loop (close to the active site) acts as a phospholipid binding site.

2. Materials and methods

2.1. Isolation of haemocyanin from Limulus polyphemus haemolymph

Haemolymph was withdrawn from the surface sterilised (70 % ethanol) cardiac sinus of healthy horseshoe crabs using 16-gauge needles (BD Microlance 3) attached to 1 mL pyrogen-free syringes. Haemolymph was centrifuged immediately at 500×*g* for 5 min (4 °C) to remove the cellular (amoebocyte) fraction. The acellular supernatant was centrifuged at 300,000×*g* for 90 min (4 °C); protein pellets were re-suspended in stabilisation buffer (100 mM Tris–HCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.5) before being applied to a calibrated Sephacryl S-500 HR gel filtration column (GE Healthcare) as described in Coates et al. (2011, 2013). Protein levels were quantified by UV absorbance measurements at 280 nm using a value of 1.39 for the absorbance of a 1 mg mL^{-1} haemocyanin solution from *L. polyphemus*, in a quartz cuvette of 1 cm pathlength. Purified haemocyanin was characterised by 280 nm: 350 nm absorption ratio values and by SDS-PAGE as previously reported (Coates et al., 2011, 2013; Supplementary Table 1). Haemocyanin (UniProt P04253) was also purchased from MERCK (formerly Sigma Aldrich, UK; H1757) as a lyophilised powder, and resuspended in 100 mM Tris–HCl pH 7.5.

2.2. Haemocyanin-derived phenoloxidase assays (EC 1.10.3.1)

L-α-Phosphatidyl-L-serine from Soybean (P0474) was purchased from MERCK (formerly Sigma–Aldrich) and prepared for assays as described previously (Coates et al., 2011, 2013). Briefly, a 1 mg mL^{-1} stock solution in 100 mM Tris–HCl, pH 7.5 was sonicated at room temperature for no more than 1 h using a Decon FS 200 Frequency Sweep water bath. Sonicated phospholipids were stored at 4 °C under nitrogen gas. Phospholipid solutions were passed six times through a 0.1 μm pore syringe filter (Anotop 10, Whatman) to eliminate large unilamellar vesicles.

Haemocyanin-derived phenoloxidase activity was determined spectrophotometrically (at 20 °C) using 96-well plates (MDS VERSAmax and BMG LABTECH SPECTROstar Nano microplate readers). Each assay (100 μL volume) consisted of 2 mM dopamine hydrochloride in 100 mM Tris–HCl, pH 7.5 and *Limulus* haemocyanin at a final concentration of 1 mg mL^{-1}. Haemocyanin (Hc) was pre-incubated for 10 min with phosphatidylserine liposomes (PS, 25.6 μM) prior to the addition of dopamine. Substrate turnover was detected by observing an increase in absorbance at 475 nm arising from the formation of DOPAchrome and its derivatives. One unit (U) represents 1 μmol of DOPAchrome formed per minute per mg protein, with an absorption coefficient of 3600 M^{-1} cm^{-1}. Haemocyanin-derived phenoloxidase activity in the presence of PS and increasing concentrations of NaCl (0–500 mM) was monitored to investigate the nature of the Hc-PS interaction. Negative controls consisted of assays absent haemocyanin and/or phosphatidylserine, and, adding the phenoloxidase inhibitor phenylthiourea (PTU, 20 μM) to ensure the change in absorbance was from enzyme activity only (Wright et al., 2012). PTU was incubated for 5 min in each combination immediately prior to the addition of substrate. Changes in absorbance values due to the (auto)catalysis of dopamine in the absence of PS were routinely subtracted from assay data.

Peptide competition assays were run in a similar manner to the enzyme assays described above with one modification: each synthetic peptide (100 μM; Table 1) was pre-incubated with haemocyanin (1 mg mL^{-1}) and phosphatidylserine (25.6 μM) prior to the addition of 2 mM dopamine (to initiate the assay). Peptides were checked for solubility at physiological pH using an online tool (https://pepcalc.com/peptide-solubility-calculator.php) before being synthesised by BIOSYNTH® Ltd. UK (formerly PEPCEUTICALS Limited), with >95 % purity. The putative structural effect of amino acid rearrangement (using a random peptide generator in ExPASY) or substituting two lysines (K) for threonine (T) or glutamic acid (E) were checked using a secondary structure prediction tool (GOR4; Table 1). The position of each proline (P) residue was fixed in all peptides to avoid gross structural changes.

2.3. Fluorescence spectroscopy of haemocyanin

All experiments were recorded on a PerkinElmer LS50 spectrofluorometer (20 °C) using a quartz cuvette (1 mL capacity) with a 5 nm bandwidth for the excitation and emission, and a scan rate of 50 nm min^{-1} (see Coates et al., 2011). Intrinsic tryptophan fluorescence from 300 nm–510 nm was detected using an excitation wavelength of 290 nm. Haemocyanin (0.1 mg mL^{-1}) was pre-incubated with phosphatidylserine (25.6 μM) for 10 min prior to fluorescence measurements. Fluorescence emission maxima of haemocyanin-phosphatidylserine with increasing concentrations of NaCl (0–500 mM) were also recorded. Control experiments – using 2 μM N-acetyl-L-tryptophan – indicated that neither phosphatidylserine and/or increasing NaCl concentrations contributed to the fluorescence signals.

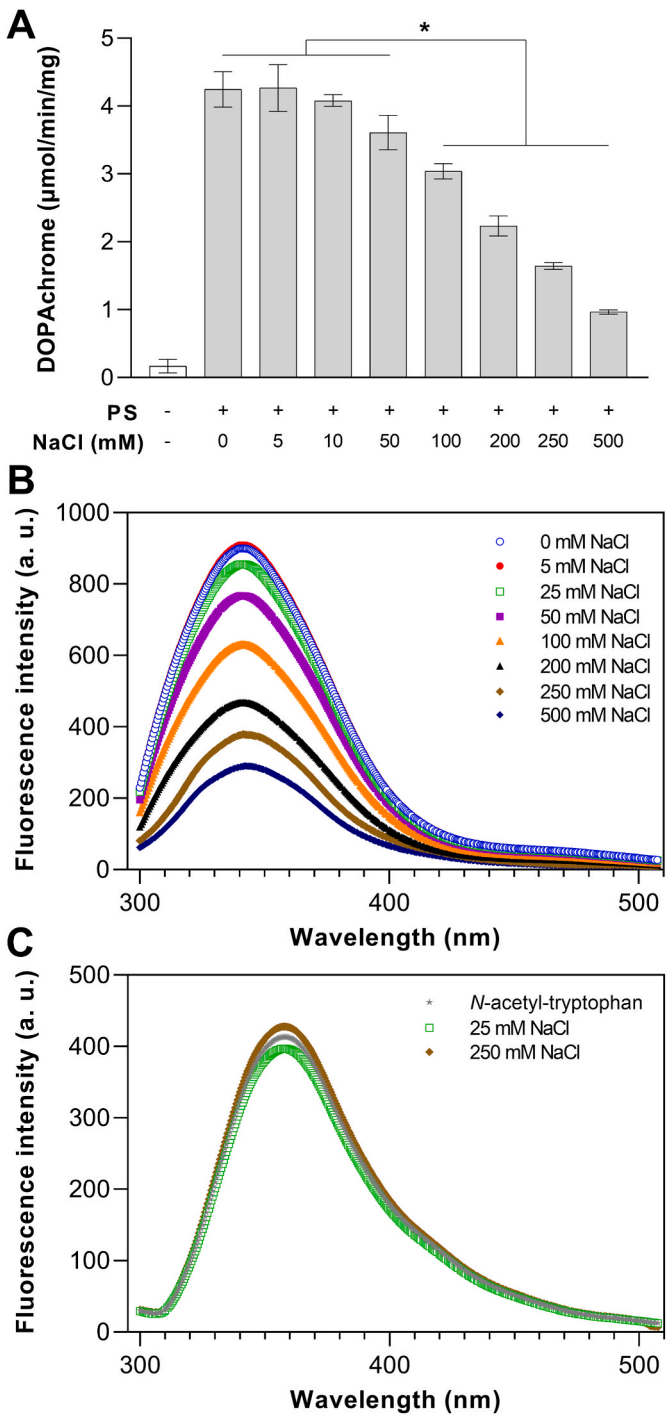

Fig. 1. (A) Phosphatidylserine induced phenoloxidase activity of hemocyanin from *Limulus polyphemus* in the presence of increasing concentrations of NaCl (0–500 mM). Hc (1 mg mL^{-1}) was pre-incubated with PS (25.6 μM) for 10 min in 100 mM Tris-HCl, pH 7.5 in the presence of increasing salt concentrations. Phenoloxidase activity was initiated by the addition of substrate (2 mM dopamine). Assays were recorded over a 6 min period. An increase in absorbance (at 475 nm) indicates the formation of DOPAchrome and its derivatives. An asterisk (*) denotes statistically significant ($P < 0.05$) differences as determined using ANOVA and multiple comparisons. **(B)** Fluorescence emission spectra of 0.1 mg mL^{-1} hemocyanin and phosphatidylserine were pre-incubated for 10 min with increasing concentrations of NaCl prior to spectral measurements. All experiments were conducted in 100 mM Tris-HCl, pH 7.5. Samples were excited at 290 nm and the subsequent fluorescent spectrum was recorded. **(C)** Fluorescence emission spectra of 2 μM N-acetyl-*L*-tryptophan (in 100 mM Tris-HCl, pH 7.5) was excited at 290 nm in the absence/presence of 25 mM and 250 mM $NaCl_2$ after 10 min incubation.

2.4. Conductivity measurements

The conductivity of 100 mM Tris-HCl, pH 7.5 in the absence and presence of increasing concentrations of NaCl (0–500 mM) was recorded at room temperature using a Portland Electronics Conductivity meter (Model P335). The fine and coarse temperature settings were 10 °C and 55 °C, respectively, using a scale of 0–30 mS. The electrode was placed in 5 mL of sample and left for 5 min to equilibrate before values were recorded.

2.5. Data handling

Haemocyanin-derived phenoloxidase assays (including peptide competition assays) were performed in triplicate (technical replicates) on three independent occasions. Data were assessed for normal distribution using the Shapiro-Wilk test and were analysed via a single variance ANOVA (with multiple comparisons). Differences were considered significant when $P \leq 0.05$. Data are expressed as the mean value ± standard error.

3. Results and discussion

The addition of NaCl to the phenoloxidase assay buffer led to a threefold increase in conductivity, from 8.7 mS with 5 mM NaCl to ≥30 mS with 500 mM NaCl (Supplementary Table 2). While no conductivity data for horseshoe crab haemolymph is available, Smith et al. (2002) recorded 389.5 mM sodium and 445 mM chloride. Herein, maximum phospholipid-induced phenoloxidase activity in haemocyanin was >4 U in the presence of 25.6 μM PS and ≤5 mM NaCl, but decreased significantly to <1 U in the presence of 500 mM NaCl ($F = 129.7$, $P < 0.0001$. Fig. 1A). The addition of NaCl to the haemocyanin-phosphatidylserine complex impacted the structural conformation as determined by a hypochromic shift in fluorescence spectra and accompanying red shift in the fluorescence emission maxima of haemocyanin around the 340 nm signal (Fig. 1B; Supplementary Fig. 1; Supplementary Table 3). Fluorescence spectra from control experiments using the model compound *N*-acetyl-L-tryptophanamide (instead of haemocyanin) demonstrated no gross effect; indicating NaCl ions did not cause a change in the fluorescence spectrum due to direct interactions with exposed tryptophan side chains (Fig. 1C). These data support a role for electrostatics (Fig. 1) in haemocyanin's interaction with PS leading to the conversion to a phenoloxidase. The phosphatidylserine-inducible phenoloxidase activity was reduced by ~75 % in the presence of the highest concentration of NaCl (>500 mM resulted in haemocyanin solubility issues). NaCl ions are known to interfere with protein stability; at low concentrations, ligand-induced ion-specific interactions of NaCl with proteins can modulate conformational stability (Date and Dominy, 2013), and this may account for the most prominent changes in the fluorescence emission maxima of haemocyanin, at concentrations above 200 mM. Increasing concentrations of NaCl can be correlated broadly with protein unfolding (Date and Dominy, 2013) – NaCl may be affecting haemocyanin and phosphatidylserine individually or together as a protein-lipid complex.

It is possible that phospholipid binding to haemocyanin is dependent on a synergy of electrostatics and direct interactions with specific amino acids at/near the dicopper active site (Fig. 2A and B). To address the latter, we synthesised several peptides based on the positively charged loop close to the dicopper active site: one wild type (WT; 181-PSTWNPKYFGKKKDRK -196), a rearranged peptide sequence with prolines fixed (RP), and two altered peptides substituting positively charged lysines with either threonine (AP1) or glutamic acid (AP2) (Table 1; Fig. 2). WT and RP had the most severe impact on haemocyanin-derived phenoloxidase activity, ≥80 % reduction in product formation (Fig. 2C; $F = 28.25$, $P < 0.001$). AP1, with a subtle change in charge (p*I* value lowered from the WT value ~10.3 to 10.01 after replacing two Ks with Ts), led to similar levels of haemocyanin-

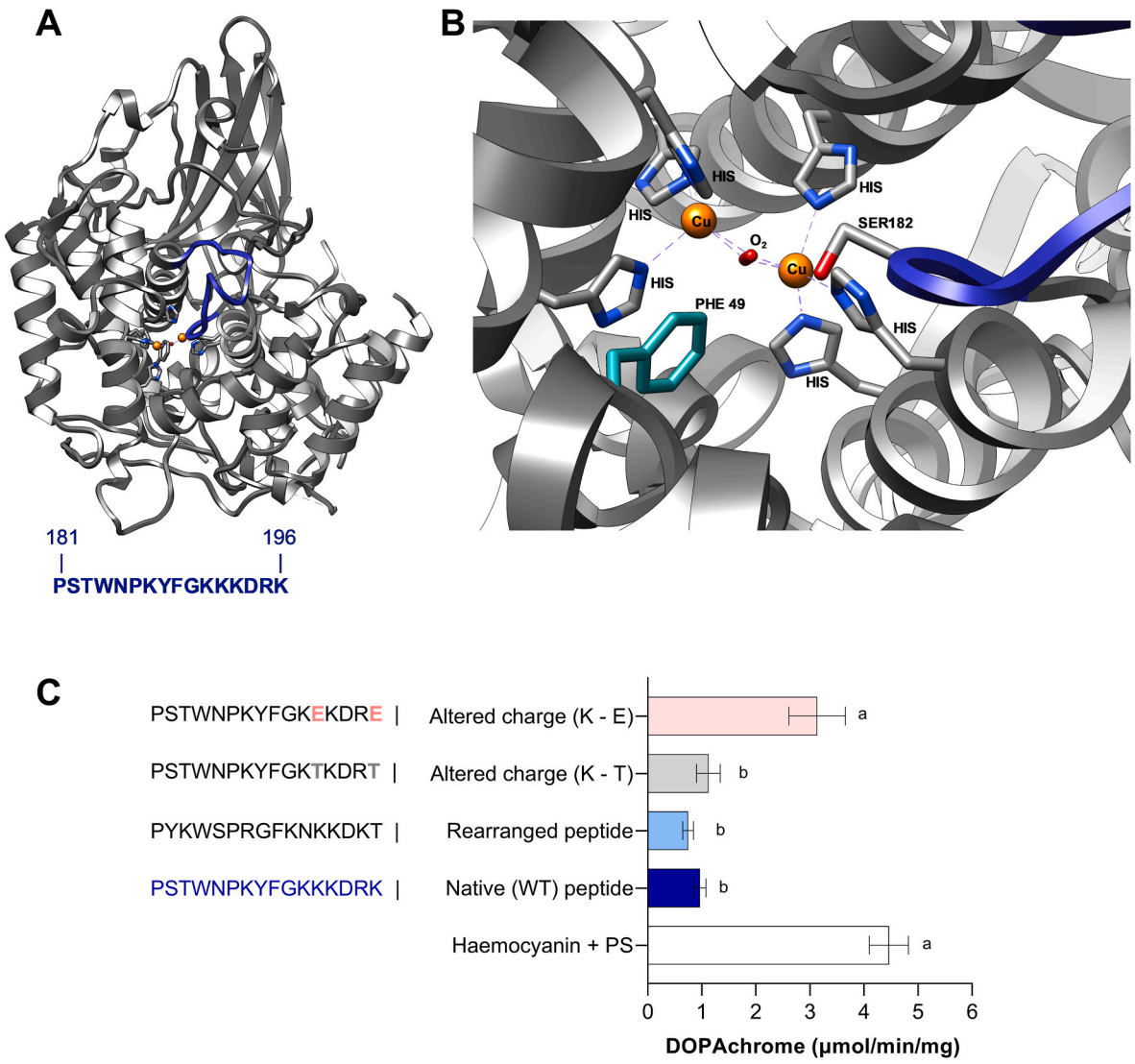

Fig. 2. (**A**) Secondary structure of *Limulus polyphemus* haemocyanin subunit II highlighting the location of the positively charged loop (blue) relative to the entrance of the dicopper active site. (**B**) Enhanced view of the proximity of the positively charged loop (notably serine 182) to the 'placeholder' reside phenylalanine 49. These two amino acids are ~7.3 Å distance from each other. PDB ID 1OXY was visualised and formatted in UCSF Chimera (Pettersen et al., 2004). (**C**) Haemocyanin-derived phenoloxidase activity in the presence of peptides representing the native (wild type sequence) loop region (i), rearranged amino acid sequence (ii), and two peptides with altered electrostatic charge via substituting two lysine residues with either two threonines (iii) or two glutamic acids (iv). Unshared letters represent statistical differences ($P < 0.05$) using one-way ANOVA and multiple comparisons.

derived phenoloxidase activity (1.12 U) to the WT (0.96 U) assay. Conversely, in the presence of AP2 with Ks substituted by Es (*pI* value lowered from ~10.3 in the WT sequence to ~8.8), haemocyanin-derived phenoloxidase activity levels were reduced to >3 U, but non-significantly ($P = 0.734$). In control assays using the enzyme inhibitor PTU (20 µM), all phosphatidylserine-induced haemocyanin-derived phenoloxidase activity was diminished by 97 % (0.14 U) to 100 %, regardless of the presence or absence of each peptide. PTU is an effective inhibitor of phenoloxidases (tyrosinase [EC 1.14.18.1], catecholoxidase [EC. 1.10.3.1]), and haemocyanin-derived phenoloxidase activity in invertebrates (Wright et al., 2012; Quinn et al., 2020). These data support that haemocyanin-phosphatidylserine interactions arise due to non-specific electrostatic interactions. Decker et al. (2001) described three *Limulus* haemocyanin subunits, namely II, V and VI, as capable of phenoloxidase activity. Upon inspection of the available sequence for subunit VI (CAJ91100.1), the corresponding region near the dicopper active site, PATWRPEVIGKVKDRK, has a theoretical p*I* of 10.28 (Mw = ~1880) – suggesting this subunit could be activated by phosphatidylserine. Previously, we identified a likely source of anionic phospholipids in horseshoe crab haemolymph – the redistributed phosphatidylserine on the outer membrane of *Limulus* immune cells (termed amebocytes) undergoing phagocytosis-induced apoptosis coincided with elevated haemocyanin-derived phenoloxidase activity (Coates et al., 2013). Incubating haemocyanin in the presence of amebocytes (5×10^5), and the apoptosis-promoting chemical roscovitine (CYC202), induced similar levels of phenoloxidase activity in haemocyanin as when using isolated phosphatidylserine liposomes.

In the context of blood coagulation, phosphatidylserine is an integral component of the catalytic surface provided by human platelets for zymogen conversion (Zwaal et al., 1998; Stace and Ktistakis, 2006). Depending on the cell type, anionic and neutral charged phospholipids can promote either procoagulant or anticoagulant reactions. According to Leventis and Grinstein (2010), when (anionic) phosphatidylserine is present in large amounts, a membrane has substantive negative electrostatic potential that attracts cations, which is "*a major mode of protein recruitment to membranes that is often overlooked*". Perhaps it is unsurprising that the nature of the phosphatidylserine-haemocyanin interaction is to a greater extent, directed by electrostatic forces. When cells

undergo apoptosis, there is a loss of asymmetrical distribution of phosphatidylserine with its redirection to the exoplasmic side of the lipid bilayer. We proposed that haemocyanin could be activated *in vivo* upon interacting with such exposed phosphatidylserine (and phosphatidylethanolamine) on apoptotic amoebocytes (Coates et al., 2013). Similarly, Bidla et al. (2009) reported that fruit fly (*Drosophila melanogaster*) clot melanisation associated with PO activity was activated by apoptotic cells, and 'super-induced' by endogenous phosphatidylserine.

In invertebrates, non-specific electrostatic interactions are linked to both cellular and humoral aspects of innate immunity. According to Wootton et al. (2006), electrostatics are crucial for haemocyte attachment, and, synergy with humoral factors for encapsulation of parasites in edible cockles, *Cerastoderma edule*. In the insect *Manduca sexta*, cleavage of the proPO zymogen into a functional PO enzyme exposes a basic region of the protein – the change in electrostatic surface potential is implicated as the trigger for the formation of an activation complex (Li et al., 2009). Alternatively, the negative surface charge of disease-causing microbes and parasites could aid them in avoiding detection by the host's immune system (Crocker and Varki, 2001).

In the absence of a 'true' phenoloxidase enzyme, chelicerates such as the Atlantic horseshoe crab, can enlist haemocyanin to catalyse the conversion of diphenols into melanin precursors. Previously, we identified phosphatidylserine as a candidate activator of haemocyanin-derived phenoloxidase, and an *in vivo* source of this anionic phospholipid on apoptotic immune cell membranes (Coates et al., 2011, 2013). Herein, we demonstrate a prominent role for non-specific electrostatics in haemocyanin associated with phosphatidylserine-induced phenoloxidase activation.

CRediT authorship contribution statement

Christopher J. Coates: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jacqueline Nairn:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization.

Declaration of AI use

We did not use AI or AI-assisted technologies at any stage in creating this article.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2025.105401.

Data availability

Data are available within the main text and online supplementary information.

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