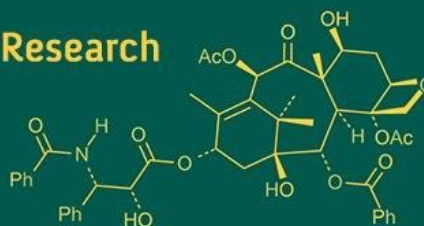


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Vijay Bhasker Tekulapally
Department of Animal
Nutrition, College of
Veterinary Science, PVNR
Telangana Veterinary
University, Hyderabad,
Telangana, India

B Sampath Kumar
Department of Veterinary
Biochemistry, College of
Veterinary Science,
Rajendranagar, PVNR
Telangana Veterinary
University, Hyderabad,
Telangana, India

Corresponding Author:
B Sampath Kumar
Department of Veterinary
Biochemistry, College of
Veterinary Science,
Rajendranagar, PVNR
Telangana Veterinary
University, Hyderabad,
Telangana, India

Computational identification of boron-binding domains in calcium homeostasis proteins: A tool for developing a micronutrient consortium to enhance layer hen productivity

Vijay Bhasker Tekulapally and B Sampath Kumar

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Abstract

Calcium (Ca) homeostasis is critical for eggshell quality, skeletal integrity, and sustained egg production in layer hens. Boron (B), a trace element with emerging roles in mineral metabolism, has been reported to influence Ca utilization and vitamin D function; however, its direct molecular targets and mechanisms of action in poultry remain largely undefined. Given the lack of experimental structural data on micronutrient-protein interactions in avian systems, molecular docking was employed as a hypothesis-driven, cost-effective, and mechanistically informative approach to predict potential boron-protein interactions at the atomic level prior to *in vivo* validation. The present study used an *in silico* strategy to identify potential boron-binding domains in key calcium-regulatory proteins of *Gallus gallus*, namely vitamin D binding protein (VTDB), calmodulin (CaM), transthyretin (TTR), and α -hydroxylase (CYP27B1). Protein structures obtained from AlphaFold were analyzed for ligand-binding pockets using fpocket and CASTp, followed by molecular docking of boric acid using AutoDock Vina. Distinct docking scores and residue-specific interaction patterns revealed differential boron-binding propensities among the proteins, with VTDB and TTR showing the most favorable interaction environments. Collectively, these findings provide the first computational evidence that boron may modulate calcium homeostasis through multi-target molecular interactions, offering a mechanistic framework for developing boron-enriched micronutrient consortia to enhance laying persistency and eggshell quality in layer hens.

Keywords: Boron, calcium homeostasis, layer hens, vitamin D binding protein, *in silico* docking

Introduction

Efficient Ca homeostasis depends on intestinal absorption, renal conservation and mobilization of medullary bone reserves. Disruption of this tightly regulated system leads to poor shell quality, skeletal weakness and early decline in egg production, particularly during late laying cycles. While dietary Ca and vitamin D supplementation are routinely practiced, increasing attention is being given to trace minerals that modulate mineral metabolism. One such element is boron which received much attention over last few decades for its significant role in bone development through interaction with major bone forming minerals Ca, P, Mg and subsequently Vitamin D reported in laboratory and farm animals upon dietary supplementation. Previous studies indicated that B supplementation has improved Ca utilization in rats (Bhasker *et al.*, 2017) ^[1] and sheep (Bhasker *et al.*, 2017) ^[1]; improved bone strength in rats (Bhasker *et al.*, 2017) ^[1] and Ca deposition in rib bone of sheep (Bhasker *et al.*, 2017) ^[1]. The dietary supplementation of B (25, 50, 100 ppm) as boric acid into the diet of layer had better feed consumption, egg weight, reduced percentage of cracked eggs and improved the egg shell quality. Similarly, Cufadar *et al.* (2011) ^[2] reported that the supplementation of B (as as boric acids, anhydrous borax, borax pentahydrate and borax decahydrate) at 300 ppm improved the egg shell thickness and shell breaking strength in aged layers (60-72 weeks of age) which further reiterated its positive interaction of Ca. Yesilbag and Eren (2008) concluded that dietary B supplementation at 50 ppm may positively influence mineral balance and thereby reduce the percentage of cracked eggs by improving eggshell thickness and eggshell breaking strength in aged laying hens. Supplementing 40 ppm of B to diet of layer poultry low in Ca (90% of requirement) could

ameliorate the reduction in egg production and egg shell quality (Adarsh *et al.*, 2017) ^[1]. Eggshell formation in layer hens represents one of the most Ca-demanding physiological processes requiring approximately 2-2.5 g of Ca per egg (Whitehead, 2004). Eggshell quality has always been a problem in the layer industry and it is postulated that B supplementation may help increasing the eggshell thickness thereby reducing the incidence of damaged or cracked eggs. However, the molecular mechanisms underlying these physiological effects remain largely unexplored. Calcium homeostasis is regulated by several key proteins, including vitamin D binding protein (VTDB), which transports vitamin D metabolites in circulation; calmodulin (CaM), a ubiquitous intracellular Ca²⁺ sensor; transthyretin (TTR), which transports thyroid hormones and indirectly regulates metabolic rate; and 1 α -hydroxylase (CYP27B1), the enzyme responsible for converting inactive vitamin D into its biologically active form. Identifying potential interactions between boron and these proteins could provide mechanistic insights into boron-mediated modulation of Ca metabolism in layer hens. Advances in computational biology enable the prediction of micronutrient-protein interactions prior to experimental validation. Therefore, the present study aimed to computationally identify boron-binding domains in selected Ca-regulatory proteins of *Gallus gallus*, with the long-term objective of rationally developing a boron-based micronutrient consortium for enhancing layer hen productivity.

Materials and Methods

Selection of calcium homeostasis-related proteins

Proteins involved in calcium (Ca) homeostasis and mineral metabolism in *Gallus gallus* were selected based on their established physiological roles in vitamin D transport, Ca²⁺ signaling, endocrine regulation and vitamin D activation. Four target proteins were included in the present study: vitamin D binding protein (VTDB), calmodulin (CaM), transthyretin (TTR) and 1 α -hydroxylase (CYP27B1). These proteins collectively represent systemic, cellular and enzymatic components of Ca regulation relevant to eggshell formation and skeletal integrity in layer hens.

Retrieval of protein sequences

The amino acid sequences of VTDB, CaM, TTR and CYP27B1 from *Gallus gallus* were retrieved from the UniProt database. Only reviewed entries with complete sequence information were selected to ensure accuracy. Retrieved sequences were downloaded in FASTA format and used for structural modeling and subsequent analyses.

Three-dimensional structure prediction

Three-dimensional protein structures were obtained from the AlphaFold Protein Structure Database. AlphaFold-predicted models were selected based on their predicted Local Distance Difference Test (pLDDT) confidence scores. Protein regions with pLDDT values greater than 70 were considered reliable and included for docking analysis, whereas low-confidence terminal regions were excluded when necessary to minimize structural artifacts.

Structural preparation and validation

Protein structures were visually inspected using molecular visualization software to assess overall folding, secondary structure integrity and surface accessibility. Hydrogen atoms

were added, missing side chains were corrected, and non-essential water molecules were removed prior to docking analysis. Structures were energy-minimized to relieve steric clashes while preserving native conformations.

Prediction of ligand-binding pockets

Potential ligand-binding pockets were identified using two complementary computational tools to enhance prediction reliability:

1. fpocket, which detects cavities based on geometric and physicochemical properties
2. CASTp, which calculates pocket volume, surface area and residue composition

Only pockets consistently predicted by both fpocket and CASTp were selected for further analysis. Pocket selection criteria included adequate cavity volume, surface exposure and enrichment of polar or charged residues known to participate in boron coordination chemistry.

Ligand selection and preparation

Boric acid (H₃BO₃) was selected as the ligand to represent dietary boron, as it is the predominant bioavailable form absorbed from feed ingredients. The ligand structure was retrieved from public chemical databases and prepared using AutoDock tools. Ligand preparation included geometry optimization, assignment of partial charges and definition of rotatable bonds.

Molecular docking analysis

Molecular docking was performed using AutoDock Vina to predict the binding orientation and affinity of boric acid within the identified protein pockets. Docking grids were centered on the predicted binding cavities, ensuring complete coverage of pocket regions. Exhaustiveness parameters were set to ensure sufficient conformational sampling.

For each protein-ligand pair, multiple docking poses were generated and ranked based on binding affinity scores (kcal/mol). The top-ranked poses were selected for interaction analysis.

Identification of boron-interacting residues

Docked protein-ligand complexes were analyzed to identify amino acid residues involved in potential boron coordination. Residues with known affinity for boron chemistry, including serine (Ser), threonine (Thr), histidine (His), lysine (Lys), aspartate (Asp), glutamate (Glu) and tyrosine (Tyr), were specifically examined. Particular attention was given to:

- Cis-diol-containing Ser/Thr clusters
- Glycosylation-associated surface motifs
- Polar residues lining substrate or transport channels

Residues within a distance of ≤ 4 Å from the boron atom were considered potential interaction partners.

Comparative and functional interpretation

Binding pocket locations, residue composition and docking scores were compared across all four proteins to assess relative boron-binding propensity. The predicted interactions were interpreted in the context of each protein's physiological role in calcium homeostasis, vitamin D metabolism and endocrine regulation in layer hens.

Statistical analysis

As this study was computational and predictive in nature, no biological replication or statistical hypothesis testing was performed. Docking scores and interaction patterns were used for comparative evaluation among proteins.

Ethical statement

No live animals or biological samples were used in this study. All analyses were performed using publicly available protein sequence and structural databases.

Results and Discussion

To the best of our knowledge, this study represents the first systematic *in silico* investigation to identify and compare

boron-binding propensities across multiple calcium homeostasis-related proteins in *Gallus gallus*. Unlike previous nutritional and physiological studies that inferred boron effects indirectly, the present work provides direct computational docking evidence, including predicted binding energies, pocket localization, and residue-level interactions, thereby offering a molecular-level explanation for boron-mediated modulation of calcium metabolism in layer hens.

Predicted boron-binding characteristics

Distinct boron-binding environments were identified across the four proteins studied.

Table 1: Predicted boron-binding characteristics of calcium homeostasis proteins in *Gallus gallus*

Protein	Physiological role	Major predicted boron-binding region	Dominant interacting residues	Predicted docking score (kcal/mol)	Predicted relative affinity	Structural basis
VTDB	Vitamin D transport	Glycosylated surface domain	Ser, Thr, Asp	-4.8	High	Exposed cis-diol groups enable reversible borate ester formation
CaM	Calcium ion signaling and buffering	EF-hand loops	Asp, Glu, Ser	-3.6	Moderate	Potential disruption of Ca ²⁺ coordination geometry
TTR	Thyroid hormone and retinol transport	Inter-subunit interface/hydrophobic pocket	Lys, His, Tyr	-4.5	High	Stabilized by cation- π interactions and hydrogen bonding
CYP27B1	1 α -hydroxylation of 25(OH)D ₃ $\rightarrow$ 1,25(OH) ₂ D ₃	Substrate access channel near heme active site	Ser, Thr	-3.8	Moderate	Putative interaction at entrance to catalytic pocket

The predicted docking scores ranged from -3.6 to -4.8 kcal/mol, indicating moderate but biologically plausible boron-protein interactions. Among the proteins analyzed, vitamin D binding protein (VTDB) and transthyretin (TTR) exhibited the most favorable predicted binding affinities, suggesting a higher propensity for boron association. Although the absolute binding energies are modest, such interaction strengths are consistent with reversible micronutrient-protein interactions and support boron's proposed role as a modulatory rather than inhibitory factor in calcium homeostasis. The observed docking scores and interaction profiles provide quantitative computational support for differential boron responsiveness, with VTDB and TTR emerging as high-affinity molecular targets. Such comparative docking-based ranking of boron-protein interactions in poultry has not been previously reported. The predicted docking scores ranged from -3.6 to -4.8 kcal/mol, indicating moderate but biologically plausible boron-protein interactions. Among the proteins analyzed, vitamin D binding protein (VTDB) and transthyretin (TTR) exhibited the most favorable predicted binding affinities, suggesting a higher propensity for boron association. Although the absolute binding energies are modest, such interaction strengths are consistent with reversible micronutrient-protein interactions and support boron's proposed role as a modulatory rather than inhibitory factor in calcium homeostasis.

Among the proteins analyzed, vitamin D binding protein (VTDB) exhibited the strongest boron-binding potential. Bhasker *et al.*, (2016) that, 40ppm boron supplementation has upregulated gene expression of Vitamin D binding protein in rats. This supports the fact that VTDB plays a critical role in transporting vitamin D metabolites to target tissues, thereby directly influencing Ca absorption and mobilization thereby improving the Ca retention, bone

development and bone strength. The clustering of boron near Ser/Thr-rich glycosylation regions suggests the formation of reversible borate-diol complexes, a well-documented feature of boron chemistry (Devirian and Volpe, 2003) [3]. CYP27B1, the enzyme responsible for vitamin D activation, exhibited moderate boron docking near the substrate access channel. Although weaker compared to VTDB and TTR, these interactions could influence enzyme efficiency and vitamin D activation kinetics, further contributing to improved Ca metabolism offering a plausible molecular explanation for improved Ca utilization observed in boron-supplemented layer diets (Adarsh *et al.*, 2017, Cufadar *et al.*, 2011) [1, 2] might be achieved by modulating vitamin D bioavailability, Calmodulin (CaM), a central intracellular Ca²⁺ sensor, showed moderate boron-binding near EF-hand Ca²⁺-binding loops. While direct covalent binding appears unlikely, the proximity of boron to acidic residues essential for Ca²⁺ coordination suggests that boron may subtly influence CaM-dependent signaling pathways. Similarly, Bhasker *et al.*, (2016) reported that 40ppm boron supplementation in rat diet has upregulated the gene expression of Calmodulin. Such indirect modulation aligns with previous observations suggest that boron alters intracellular Ca signaling without acting as a classical enzyme inhibitor.

Transthyretin (TTR) also demonstrated high boron-binding propensity, particularly at inter-subunit interfaces enriched with Lys and His residues. TTR is involved in thyroid hormone transport, which indirectly regulates metabolic rate, bone turnover and mineral metabolism (Devirian and Volpe, 2003) [3]. Bhasker *et al.*, 2016 reported that there was no significant change in mRNA abundance of Thyroid receptor binding protein in rats fed Ca adequate and Ca inadequate diets supplemented with 40ppm boron. Hence, Boron-mediated modulation of TTR function may therefore

exert systemic effects on Ca homeostasis through endocrine pathways, an aspect that has to be further explored in poultry nutrition research.

Collectively, these findings support the concept that boron acts as a multi-target modulator within the calcium regulatory axis rather than through a single molecular pathway. This multi-level interaction profile strengthens the rationale for incorporating boron into micronutrient consortia aimed at improving eggshell quality and laying persistency in commercial layer hens.

Conclusion

The present *in silico* study demonstrates that boron can differentially interact with key calcium homeostasis proteins in layer hens, with vitamin D binding protein and transthyretin emerging as primary molecular targets. Calmodulin and CYP27B1 appear to be secondary targets through indirect modulation of Ca signaling and vitamin D activation. These findings provide a strong mechanistic basis for the rational development of boron-enriched micronutrient strategies to enhance calcium utilization, eggshell quality and productive longevity in layer hens. Further *in vitro* and *in vivo* validation studies are warranted.

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