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Inhibitory Effects of Chitosan-Silver Nanoparticles on Acetylcholinesterase from the Black Bean Aphid

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Abstract

This study examined chitosan recovered from fish-scale waste, the synthesis of chitosan-silver nanoparticles (Ch-AgNPs), and their inhibition of acetylcholinesterase (AChE) from the black bean aphid, Aphis fabae. Fish scales were demineralized, deproteinized, and deacetylated under different solid-to-alkali ratios and temperatures. The resulting material and Ch-AgNP preparations were characterized by yield, pH, viscosity, water-binding capacity, fat-binding capacity, degree of deacetylation, and Fourier-transform infrared spectroscopy. The highest recorded chitosan yield (46.686%) occurred at a fish-scale-to-NaOH ratio of 1:2 and 55 °C. The degree of deacetylation ranged from 21.533% to 57.593%. Aphid AChE was purified by ammonium sulfate precipitation, DEAE-52 ion-exchange chromatography, and Superdex 200 gel filtration. The final preparation had a specific activity of 24.53 U mg−1, a reported purification fold of 229.40, and a recovery of 13.99%. Crude and purified AChE showed temperature optima near 30 °C; their pH optima were 7.0 and 7.5, respectively. Ch-AgNP preparations inhibited both enzyme preparations, although the IC50 pattern was not monotonic across the five formulations. These findings support further evaluation of fish-scale-derived Ch-AgNPs as enzyme-targeted materials, with additional nanoparticle characterization and bioassays needed before pesticidal efficacy can be established.

INTRODUCTION

Green synthesis offers a practical route to metal nanoparticles while reducing reliance on harsh reagents and energy-intensive processing. Silver nanoparticles (AgNPs) are of particular interest because their surface-dependent properties support antimicrobial, catalytic, and material applications. Biopolymer-assisted synthesis can also stabilize the particles and introduce functional groups that influence their interactions with biological targets [1,2]. Chitosan is produced by partial deacetylation of chitin, a structural polysaccharide abundant in crustacean shells, fish scales, and fungal cell walls. Its amino and hydroxyl groups contribute to its biodegradability, biocompatibility, adsorption capacity, and ability to coordinate metal ions. These properties have encouraged the use of chitosan in drug delivery, tissue engineering, water treatment, food coatings, and composite materials [3,4]. Fish-processing waste is therefore a potential low-cost source of chitosan and provides an opportunity to convert an underused residue into a functional biomaterial [3].

Combining chitosan with silver can yield nanocomposites with biological activity distinct from that of either component alone. Chitosan may act as a reducing, capping, or stabilizing matrix, while silver contributes strong surface reactivity. Chitosan-based silver nanocomposites have shown antifungal and antibacterial activity, but their effects depend on composition, particle size, surface charge, and the test organism [1,2].

Acetylcholinesterase (AChE; EC 3.1.1.7) terminates cholinergic signalling by hydrolysing acetylcholine to choline and acetate (**Fig. 1**). In insects, inhibition of AChE causes acetylcholine to accumulate at synapses, producing sustained stimulation and, ultimately, neuromuscular failure. This enzyme is consequently the principal biochemical target of organophosphate and carbamate insecticides and is widely used in inhibitive assays [5,6].

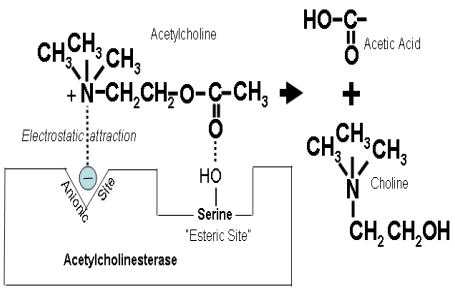

Figure 1. Breakdown of acetylcholine.

The black bean aphid, Aphis fabae, feeds in colonies on stems, leaves, and growing points. Heavy infestations weaken plants through sap removal, and aphids can also contribute to the spread of plant viruses. Dependence on conventional insecticides raises concerns about resistance, residues, and effects on non-target organisms. Enzyme-directed materials produced from biological waste may therefore provide useful leads for lower-impact pest-management approaches. This study investigated the physicochemical properties of fish-scale-derived chitosan and Ch-AgNP formulations, purified AChE from A. fabae, and assessed inhibition of the crude and purified enzymes.

MATERIALS AND METHODS

Reagents

Sodium hydroxide (NaOH), hydrochloric acid (HCl), silver nitrate (AgNO3), ascorbic acid, and glacial acetic acid were of analytical grade and obtained from Sigma-Aldrich. DEAE-52, Superdex 200, and protein standards for polyacrylamide-gel electrophoresis were obtained from Shanghai Runcheng Bioscience. Molecular-mass markers (14.4-97.4 kDa) were obtained from Sigma Chemical Company.

Sample collection

Fish scales were collected from a fish market in Katsina State and transported to the laboratory at Umaru Musa Yar'adua University for processing. A laboratory colony of Aphis fabae was maintained on broad bean plants at 25 ± 5 °C and 65 ± 5% relative humidity under a 16 h light:8 h dark photoperiod. Aphids were collected with an aspirator and stored at −20 °C until enzyme extraction.

Extraction of Chitosan

Chitosan was prepared through demineralization, deproteinization, and deacetylation [3,7]**.** Fish scales were washed to remove adhering tissue, sun-dried, and allowed to undergo autolysis for 24 h at room temperature (**Fig. 2**). They were then treated with 2% HCl at a solid-to-solvent ratio of 1:5 (w/v) for 16 h at room temperature. The residue was washed to neutral pH and dried. Deproteinization was carried out with 4% NaOH at a solid-to-solvent ratio of 1:5 (w/v) for 20 h at room temperature (**Fig. 3**). The residue was washed to neutral pH and sun-dried.

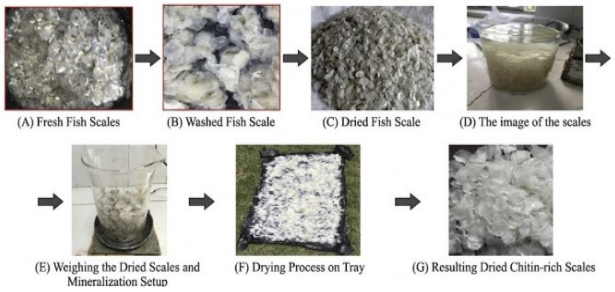

Fig. 2. Laboratory-scale processing of fish scales: (A) collection; (B) washing; (C) drying; (D) bulk material; (E) weighing and acid demineralization; (F) drying of the chitin-rich residue; and (G) recovered dried residue.

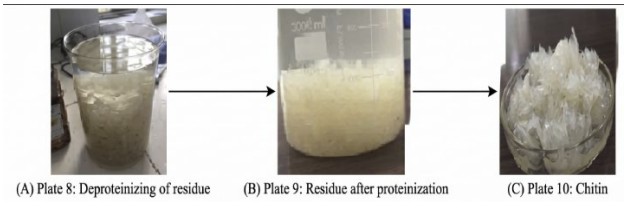

Fig. 3. Deproteinization during chitin isolation: (A) scales treated with NaOH; (B) residue after alkaline treatment; and (C) deproteinized chitin washed to neutral pH.

Deacetylation was performed with 4% NaOH at 60 ± 5 °C and a solid-to-solvent ratio of 1:10 (w/v) for 20 h (**Fig. 4**). The residue was washed and oven-dried at 40 ± 5 °C for 4 h.

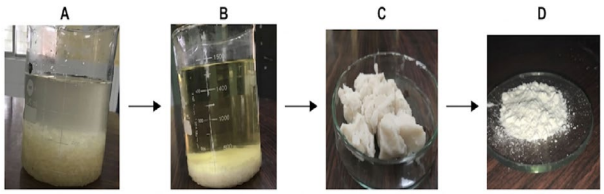

Fig. 4. Conversion of chitin to chitosan: (A) alkaline deacetylation at 60 ± 5 °C; (B) post-reaction mixture; (C) washed, neutralized residue; and (D) dried and ground chitosan.

Determination of percentage yield

Percentage yield was calculated as the mass of dried chitosan divided by the initial mass of fish scales, multiplied by 100.

$$Yield(\%) = \frac{\text{Weight of Chitosan (g)}}{\text{Weight of initial dry scale used (g)}} \text{X } 100$$

Synthesis of chitosan-silver nanoparticles

Chitosan (0.5 g) was added separately to 125 mL portions of silver nitrate solution at nominal concentrations of 1%, 2%, 3%, 4%, and 5% and heated to 90 °C. Sodium hydroxide (5 mL, 0.25 M) was added with stirring.

Formation of Ch-AgNPs was indicated by a colour change from colourless to dark brown over 15 min (**Fig. 5**). Each mixture was cooled and filtered through Whatman grade 1 filter paper. The recovered solid was washed three times with distilled water, air-dried, and stored for analysis [2].

Fig. 5. Chitosan-silver nanoparticle preparations synthesized using different nominal silver nitrate concentrations.

Determination of pH
The pH was measured with a calibrated digital pH meter after dispersing 1 g of chitosan or Ch-AgNP material in 10 mL of distilled water.

Determination of the degree of deacetylation
The degree of deacetylation was determined by acid-base titration. Chitosan (0.2 g) was dissolved in 20 mL of 0.1 M HCl and 25 mL of distilled water, and the solution was titrated with 0.1 M NaOH. The degree of deacetylation was calculated using Equation 2.

$$Deacetylation\ Degree\ (\%) = \frac{2.03(V2 - V1)}{m + 0.0042(V2 - V1)}$$

Determination of Ch-AgNP viscosity
Viscosity was measured at room temperature with a rotational viscometer (Viscostar+ L, Kinematica AG, Switzerland), using spindle 4 at 20 rpm.

Extraction of AChE from black bean aphids
Aphid heads and appendages were homogenized on ice in 0.1 M sodium phosphate buffer (pH 7.5) containing 2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 10,000 × g for 1 h at 4 °C, and the supernatant was retained for the AChE assay and subsequent purification.

Determination of acetylcholinesterase activity
AChE activity was measured by a microplate adaptation of the Ellman method [8]. Enzyme preparation (20 µL) was mixed with 20 µL of 0.6 mM acetylthiocholine iodide and incubated at 30 °C for 10 min. The reaction was initiated by adding 180 µL of 0.4 mM 5,5′-dithiobis(2-nitrobenzoic acid), and absorbance at 420 nm was monitored for 20 min. Measurements were performed in triplicate.

Determination of protein concentration
Protein concentration was determined by the Bradford method, using bovine serum albumin as the standard [9].

Preparation of the crude enzyme extract
Approximately 1 g of adult aphid heads and appendages was homogenized in 15 mL of 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA, 1% Triton X-100, and 1 M NaCl. The homogenate was filtered through glass fibre and centrifuged at 12,000 × g for 30 min at 4 °C. Solid ammonium sulfate was added gradually to the supernatant to 45% saturation with gentle stirring on ice. After 20 min, the suspension was centrifuged under the same conditions. The pellet was dissolved, dialysed against distilled water, and concentrated with PEG 6000 before ion-exchange chromatography [10].

Chromatography on DEAE-52
A DEAE-52 column was equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 1% Triton X-100. The dialysed sample (8 mL) was applied, and bound proteins were eluted with a 0-0.5 M NaCl gradient in phosphate buffer at 0.5 mL min−1 and 4 °C. Fractions containing AChE activity were pooled [10].

Chromatography on Superdex 200
Active fractions from the DEAE-52 step were applied to a Superdex 200 column (65 × 1.1 cm) equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 0.4 M NaCl. Elution was performed at 0.5 mL min−1 and 4 °C, and the most active fractions were pooled [10].

Calculation of enzyme activity, specific activity, purification fold, and recovery
One unit of AChE activity was defined as the amount of enzyme catalysing the conversion of 1 µmol of substrate per minute under the assay conditions. Specific activity was expressed as units per milligram of protein. Purification fold was calculated relative to the specific activity of the crude extract, and recovery was calculated as the total activity after each step divided by the initial total activity, multiplied by 100.

Effects of temperature, pH, and substrate concentration
AChE activity was examined at temperatures of 5-75 °C, pH values of 5.0-9.0, and acetylthiocholine iodide concentrations of 250-1,200 µM.

Native PAGE and SDS-PAGE
Native PAGE and SDS-PAGE were performed with 4% stacking and 12% resolving gels. Samples were run at 80 V through the stacking gel and 120 V through the resolving gel. Native PAGE was conducted at 4 °C and SDS-PAGE at room temperature. Molecular-mass standards (14.4-97.4 kDa) were included, and gels were stained with Coomassie Brilliant Blue R-250.

In vitro inhibition of black bean aphid AChE by Ch-AgNPs
Crude and purified AChE preparations were pre-incubated with each Ch-AgNP formulation at 37 °C for 30 min. Residual activity was measured, and IC50 values were estimated by regression analysis of the inhibition data.

Data analysis
Data from experiments conducted in triplicate were analysed by one-way analysis of variance. Means were compared using the least significant difference test at $p \leq 0.05$ (Minitab version 15.0).

RESULTS

Chitosan yield varied with both the fish-scale-to-NaOH ratio and deacetylation temperature (**Table 1**). Across the conditions tested, the yield ranged from 10.666% to 46.686%. At each ratio, yield increased as temperature rose from 37 to 55 °C; conversely, yield generally declined as the amount of NaOH relative to fish scales increased. The highest yield was recorded at a ratio of 1:2 and 55 °C.

Physicochemical properties of chitosan and Ch-AgNP formulations
The Ch-AgNP formulations differed significantly in viscosity ($p \leq 0.05$; **Table 2**). Viscosity increased from 888.75 ± 5.657 mPa·s for the 1% formulation to 5,847.3 ± 20.1101 mPa·s for the 5% formulation. The pH, water-binding capacity, and fat-binding capacity decreased across the same series. These trends show that formulation composition affected several bulk physicochemical properties.

Table 1. Percentage yield of chitosan isolated from fish scale. Different superscripts in the same column indicate statistical difference ($p \leq 0.05$) (n=3).

Fish scale:NaOH ratio (w/v)	Deacetylation temperature (°C)	Yield (%)
1:2	37	26.933±1.708[a]
	45	36.760±1.446[b]
	55	46.686±0.956[c]
1:4	37	22.540±1.040[a]
	45	25.493±0.985[a]
	55	30.816±0.741[b]
1:6	37	20.516±0.266[a]
	45	22.659±0.525[a]
	55	24.633±.2082[b]
1:8	37	18.600±0.078[c]
	45	20.593±0.162[c]
	55	21.816±0.127[c]
1:10	37	10.666±0.076[d]
	45	13.576±0.086[d]
	55	15.742±0.127[e]

Note: Different superscripts in the same column indicate statistical difference ($p \leq 0.05$)

Table 2. Physicochemical parameters of synthesized chitosan silver nanoparticles. Different superscripts in the same column indicate statistical difference ($p \leq 0.05$) (n=3).

Sample	pH	Viscosity (mPa·s)	WBC (%)	FBC (%)
Chitosan	9.4667±0.1528[a]	6543.87±23.894	580.55±25.054	520.56±15.056
1% Ch-AgNPs	8.3333±0.1124[a]	888.75 ± 5.657[a]	560.34±22.401[a]	462.24±12.423[a]
2% Ch-AgNPs	7.8667±0.1628[b]	1488.75 ± 8.456[b]	520.56±20.350[b]	405.32±11.302[b]
3% Ch-AgNPs	6.7667±0.1728[b]	2481.8 ± 12.546[c]	460.34±21.250[c]	395.34±9.601[c]
4% Ch-AgNPs	5.5667±0.2528[b]	4806.7 ± 15.7890[d]	405.12±23.230[d]	325.35±12.401[d]
5% Ch-AgNPs	4.4320±0.0528[b]	5847.3 ± 20.1101[e]	360.32±19.410[e]	298.34±8.101[e]

Note: Different superscripts in the same column indicate statistical difference ($p \leq 0.05$)

Water-binding capacity ranged from 360.32 ± 19.410% to 560.34 ± 22.401% among the Ch-AgNP formulations (**Table 2**). The values fall within the broad range previously reported for chitosan materials, although direct comparisons should account for differences in source material and processing conditions [3,7].

Degree of deacetylation

The degree of deacetylation ranged from 21.533 ± 1.724% to 57.593 ± 1.527%. These values were below the 70-95% range commonly associated with commercial chitosan, indicating incomplete deacetylation under the relatively mild alkaline and temperature conditions used here [3,7]. Stronger alkali, higher temperatures, or longer reaction times may be required to increase deacetylation.

Fourier-transform infrared spectroscopy

The FTIR spectrum of the isolated material is shown in **Fig. 6**. The band at 3,423.83 cm−1 was assigned to O-H stretching, the band at 1,417.69 cm−1 to CH2 bending, and the bands at 1,032.53 and 873.89 cm−1 to C-O-related vibrations. The profile was consistent with the principal functional groups expected in chitosan [3].

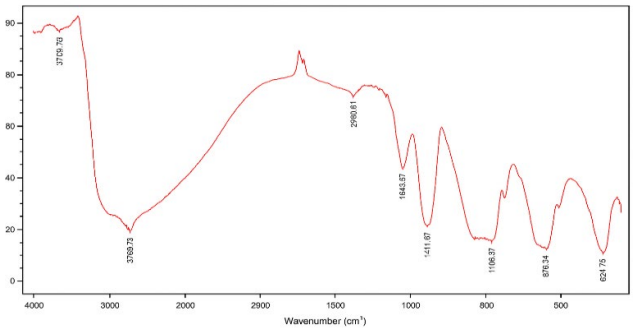

Fig. 6. FTIR spectrum of chitosan isolated from fish scales.

AChE was purified by ammonium sulfate precipitation, dialysis, DEAE-52 ion-exchange chromatography, and Superdex 200 gel filtration (**Table 3**; **Figs. 7** and **8**). The final fraction had a specific activity of 24.53 U mg−1, a reported purification fold of 229.40, and a recovery of 13.99%. Electrophoresis showed one major band under denaturing conditions and three bands under native conditions (**Fig. 9**).

Table 3. Results of the purification of acetylcholinesterase from black bean aphids.

Purification step	Volume (mL)	Total protein (mg)	Specific activity (U mg^{-1})	Total activity (U)	Purification (fold)	Recovery (%)
Crude extract	15	252.70	0.11	26.21	1.00	100.00
(NH4)2SO4 precipitation	8	56.56	0.23	13.47	2.34	52.16
Dialysis	10	41.77	0.24	10.26	2.46	39.36
DEAE-52	8	0.52	10.63	6.31	100.34	23.21
Superdex 200	3	0.12	24.53	3.70	229.40	13.99

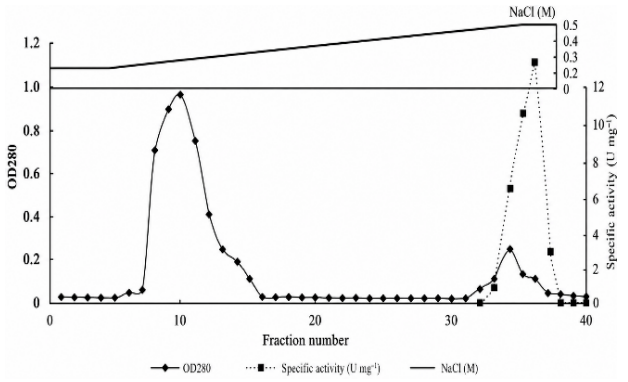

Fig. 7. Elution profile of black bean aphid AChE from DEAE-52 chromatography, showing absorbance at 280 nm and AChE activity.

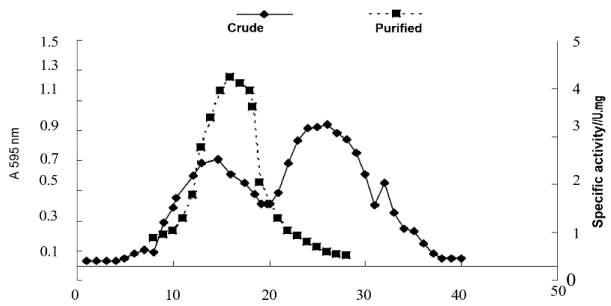

Fig. 8. Elution profile of black bean aphid AChE from Superdex 200 chromatography, showing absorbance at 280 nm and AChE activity.

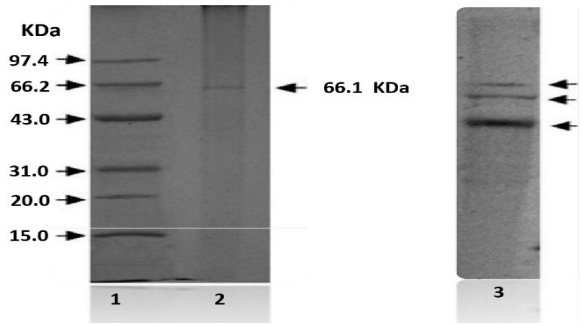

Fig. 9. Electrophoretic analysis of purified black bean aphid AChE. Lane 1, molecular-mass markers; lane 2, purified AChE under denaturing SDS-PAGE conditions; lane 3, purified AChE under native PAGE conditions. Gels were stained with Coomassie Brilliant Blue R-250.

AChE activity under different temperature conditions

Activities of both crude and purified AChE increased between 5 and 30 °C and reached maxima at approximately 30 °C (**Fig. 10**). Activity subsequently declined as temperature increased, consistent with thermal disruption of enzyme structure and catalysis [12].

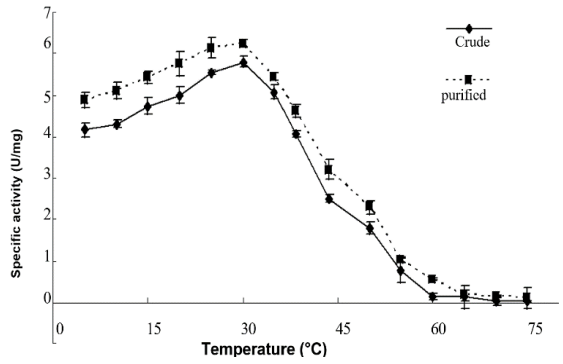

Fig. 10. Effect of temperature on the activities of crude and purified AChE from black bean aphids.

The pH profiles of the two preparations differed slightly (**Fig. 11**). Crude AChE showed maximum activity at pH 7.0, whereas purified AChE was most active at pH 7.5. Activity declined on either side of these optima.

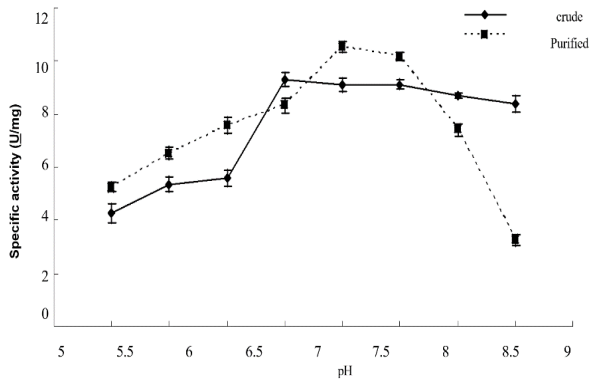

Fig. 11. Effect of pH on the specific activities of crude and purified AChE from black bean aphids.

Crude AChE reached maximum activity at 600 µM acetylthiocholine iodide, whereas purified AChE reached its maximum at 700 µM (**Fig. 12**). Activity declined at higher substrate concentrations, suggesting substrate inhibition under the assay conditions.

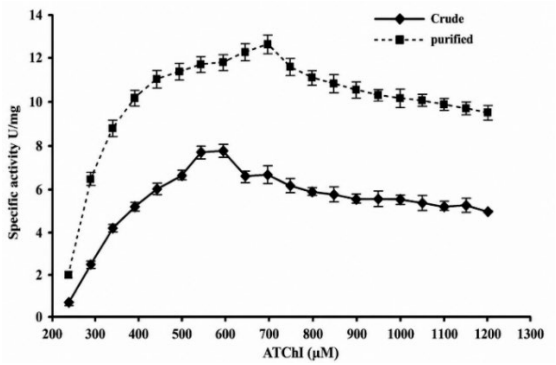

Fig. 12. Effect of acetylthiocholine iodide concentration on the specific activities of crude and purified AChE from black bean aphids.

All five Ch-AgNP formulations inhibited crude and purified AChE (**Table 4**). Based on the reported IC50 values, the crude-to-purified IC50 ratio ranged from 0.095 to 1.18. The response was not monotonic across formulations; therefore, increasing the nominal silver nitrate concentration used during synthesis did not produce a uniform increase in inhibitory potency.

Table 4. In vitro inhibition of black bean aphid acetylcholinesterase by Ch-AgNP formulations.

	Crude AChE IC50 (mol L^{-1})	Purified AChE IC50 (mol L^{-1})	IC50 ratio Crude/purified
1%Ch-AgNPs	1.76×10^{-5}	1.18×10^{-4}	0.149
2%Ch-AgNPs	2.76×10^{-4}	2.34×10^{-4}	1.18
3%Ch-AgNPs	4.12×10^{-4}	4.05×10^{-4}	1.02
4%Ch-AgNPs	5.94×10^{-4}	6.72×10^{-4}	0.884
5%Ch-AgNPs	7.76×10^{-5}	8.134×10^{-4}	0.095

Note: Ch-AgNPs, chitosan-silver nanoparticles; IC50, concentration producing 50% inhibition.

DISCUSSION

The purification sequence enriched AChE substantially but recovered only 13.99% of the initial activity. Losses during ammonium sulfate precipitation, dialysis, and chromatography are expected because each step removes non-target proteins and may also reduce enzyme stability. The marked increase in specific activity after DEAE-52 and Superdex 200 chromatography indicates effective enrichment, although the numerical purification factors should be interpreted alongside the rounded protein and activity values reported in **Table 3**. Similar combinations of salt fractionation, ion exchange, and gel filtration have been used for insect AChE purification [10,13]. The single major SDS-PAGE band near 66 kDa suggests a predominant polypeptide of that apparent molecular mass. In contrast, the three bands observed by native PAGE may represent oligomeric or differently associated forms of AChE.

Multiple native forms have been reported for insect cholinesterases, but their identity in *A. fabae* cannot be confirmed from electrophoretic mobility alone [10,13]. Additional analyses, such as activity staining, size-exclusion calibration, or mass spectrometry, would be needed to establish the composition of these bands.

The temperature and pH optima were consistent with an enzyme adapted to moderate physiological conditions. The decline in activity above 30 °C is compatible with temperature-dependent loss of catalytic structure [12]. Ch-AgNPs inhibited both crude and purified AChE, but the IC50 values did not follow a simple concentration-response pattern across formulations. Because the nominal percentages describe synthesis formulations rather than verified nanoparticle dose or silver content, the present results should be viewed as preliminary. Particle-size distribution, zeta potential, silver content, release of Ag+ ions, and whole-insect toxicity should be determined before pesticidal activity and selectivity can be concluded.

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AUTHOR CONTRIBUTIONS

Conceptualization, Data analysis, Data curation, Writing-original draft, and editing were equally shared by A.M., A.U., I.H.K., S.A.A. and E.E.

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest.

FUNDING STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

The supplementary files and raw data supporting the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL APPROVAL AND CONSENT

Not applicable

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