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Effects of a New Microbial α-Amylase Inhibitor Protein on *Helicoverpa armigera* Larvae

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ABSTRACT: A new microbial α-amylase inhibitor gene was cloned and characterized. The encoded, recombinant, α-amylase inhibitor protein was induced and expressed by isopropyl β-D-1-thiogalactopyranoside (IPTG) in *Escherichia coli* M15 cells. The effects of the α-amylase inhibitor protein on *Helicoverpa armigera* larvae were studied. Compared to the control, the weight of *H. armigera* larvae fed the diet with recombinant α-amylase inhibitor protein added at a concentration of 20 μg/g was reduced by 49.8%. The total soluble protein of *H. armigera* larvae fed the diet with the α-amylase inhibitor protein added was also reduced by 36.8% compared to the control. The recombinant α-amylase inhibitor protein showed inhibition activity against α-amylase of *H. armigera*. These results suggested that this α-amylase inhibitor protein may be a promising bioinsecticide candidate for controlling *H. armigera*.

KEYWORDS: *α-amylase inhibitor protein, microorganism, Helicoverpa armigera, larval weight*

INTRODUCTION

The cotton bollworm (*Helicoverpa armigera* Hubner) is one of the important lepidopteran pests that damage a wide range of field, floricultural, and horticultural crops. *H. armigera* larvae are polyphagous, and their host species include many important agricultural crops, such as cotton, maize, chickpea, pigeonpea, and sorghum;[1,2] they feed on these crops and cause substantial economic loss. Many studies have been conducted on methods to control this insect pest.[1−4] Due to environmental concerns with and resistance to common synthetic insecticides, natural insect controls such as insecticidal toxins from the bacterium *Bacillus thuringiensis* Berliner (BT) have been developed to control this pest.[3,4] In China, BT cotton has been commercialized since 1997 and has been effective against *H. armigera* for many years. However, there have been increasing reports that this pest has developed resistance to BT cotton.[5−8] Thus, it is necessary to search for new genes to control *H. armigera*.

The α-amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) belong to the family of endoamylases that catalyze the hydrolysis of α-D-(1−4)-glucan linkages in starch components, glycogen, and other carbohydrates. These enzymes play a key role in carbohydrate metabolism of microorganisms, plants, and animals.[9] The α-amylase inhibitors, which can inhibit the activity of insect amylases, are attractive candidates for the control of seed weevils as these insects are highly dependent on starch as an energy source. Thus far, several α-amylase inhibitors that confer resistance to insect pests have been isolated from Leguminosae plant seeds, such as pigeonpea (*Cajanus cajan*), cowpea (*Vigna ungiculata*), and common bean (*Phaseolus vulgaris*), as well as from cereals, such as wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), and rye (*Secale cereale*).[10−15] The α-amylase inhibitor purified from *Phaseolus coccineus* has strong inhibition activity against α-amylases from coffee berry borers.[16] The α-amylase inhibitors extracted from *Amaranthus hypochondriacus* seeds also display strong inhibition effects against insects.[17] The α-amylase inhibitors from wheat can inhibit the amylases from *Lygus hesperus* and *Lygus lineolari*.[18] Pueyo et al.[19] reported that α-amylase inhibitors from bean and wheat inhibit larval growth of red flour and flat grain beetles. In addition, expression of the α-amylase inhibitor from *P. vulgaris* seeds is lethal to several insects, including pea weevil and some species of bruchids in transgenic plants.[20]

However, reports on α-amylase inhibitor proteins against insect amylases from microorganisms are limited. Here, we cloned a new α-amylase inhibitor gene from *Streptomyces avermitilis* by polymerase chain reaction (PCR) and expressed the α-amylase inhibitor protein in *Escherichia coli* cells. The characteristics of this recombinant protein and its effect on *H. armigera* were investigated.

MATERIALS AND METHODS

Experimental Strain and Insects. *S. avermitilis* was kindly provided by Dr. X. Jiang, Chinese Academy of Agricultural Sciences; *E. coli* DH5α and M15 cells were used for DNA manipulation and expression of recombinant α-amylase inhibitor protein. Recombinant *E. coli* cells were grown in LB-ampicillin (100 μg/mL).

H. armigera larvae obtained from the Institute of Cotton, Chinese Academy of Agricultural Sciences, were fed an artificial diet after hatch at 28 °C in the dark. The second-instar larvae were used for feeding

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experiments, and the fifth-instar larvae were used for amylase activity analysis.

Gene Cloning. *S. avermitilis* spores were inoculated into YEME medium and cultured for 72 h by shaking at 28 °C. Then the mycelia were collected by centrifugation. The mycelia DNA was extracted using the *Streptomyces* bacterial genomic DNA extraction kit (GENMED, catalog no. GMS60026.1.2). The primers used for PCR amplification of α-amylase inhibitor were as follows: 5′-GCTGATGGCACTCCTGTTCACG-3′ and 5′-CTTCATCCGCGTCGTCAACCAG-3′.[21] PCR products were purified using an agarose gel purification kit (TIANGEN Biotech Co. Ltd., Beijing, China; catalog no. DP209). Purified PCR products were ligated into the pMD18-T vector, transformed into DH5α chemically competent cells (Qiagen, Valencia, CA, USA), and sequenced. Acquired sequences were blasted against the GenBank database. New primer pairs (5′-ATGAGGCGGTTCAGTCGTTCAA-3′ and 5′-CTACAGGCGGGGATTCGCCTCC-3′) were then designed and used to amplify the full-length α-amylase inhibitor sequence. Cycling conditions were 94 °C for 5 min, followed by 35 cycles at 94 °C for 40 s, 58 °C for 40 s, 72 °C for 1 min, and a final elongation for 10 min. The full-length sequence amplified by PCR was purified using an mini agarose gel purification kit (Tiangen), ligated into the pQE-30 vector (Qiagen), and transformed into M15 cells (Dingguo Biological Technology Limited Co., Beijing, China) for protein expression.

The sequence of the inhibitor gene was submitted to GenBank for comparison, and the sequence similarity was analyzed using MegAlign from the DNAstar software package (DNASTAR, Inc., Madison, WI, USA).

α-Amylase Inhibitor: Expression and Purification. A single M15 colony containing the pQE-30-SA-AI vector (vector containing the α-amylase inhibitor gene) was inoculated into LB/ampicillin medium and grown overnight. The bacteria were grown at 37 °C for 3 h after induction with IPTG (final concentration at 1 mM). The cultures were then centrifuged, and the pellets were washed twice and resuspended in cold PBS buffer (NaCl 0.137 mol/L, KCl 2.68 mmol/L, KH_2PO_4 1.47 mmol/L, Na_2HPO_4 8.38 mmol/L, pH 7.4). The bacteria were sonicated until a noticeable clearing of the bacterial suspension occurred. The sonicated bacteria were then centrifuged at 12000*g* for 30 min. The supernatants and pellets were then collected separately for purification and SDS-PAGE detection.

The supernatant from the former step was combined with 2.5 mL of nickel(II)−nitrilotriacetic acid (Ni-NTA) His Bind resin (Novagen, catalog no. 70666) equilibrated in the buffer (100 mmol/L NaH_2PO_4, 10 mmol/L Tris-HCl, 8 mmol/L urea, pH 8.0). The supernatant was then filtered through a 0.45 μm membrane and added to the equilibrated resin column. The column containing the sample was washed three times to remove the unbound proteins. The column was then washed with wash buffer (250 mM imidazole) to elute the specific α-amylase inhibitor protein. The purification was conducted at 4 °C.

The purified proteins from the above steps were dialyzed with a series concentration gradient of urea buffer (6, 4, 3, 2, 1, and 0.5 M) containing 1.1 mM oxidized glutathione GSH and 2.2 mM glutathione. After an overnight dialysis with PBS buffer, the protein solution was centrifuged at 12000*g* for 20 min at 4 °C. The supernatant was collected and placed at −20 °C until further use. Protein concentrations were determined by using the BCA reagent kit (Pierce, Rockford, IL, USA) and BSA (Sigma-Aldrich, St. Louis, MO, USA) as a standard. The purified protein was further analyzed by SDS-PAGE.

SDS-PAGE. The purified recombinant α-amylase inhibitor protein was separated by SDS-PAGE to analyze the purity and relative molecular weight of the protein. Twenty micrograms of purified protein was separated using SDS-PAGE (5% stacking gel and 15% separating gel) for 15 min at 80 V and 45 min at 180 V with a Bio-Rad Mini-Protein III cell system (Bio-Rad Laboratories, Hercules, CA, USA). The gels were stained with Coomassie brilliant blue R-250.

Analysis of α-Amylase Activity and Effects of Inhibition. The inhibition of α-amylase activities of *H. armigera* larvae was determined. For sample preparation, 10 fifth-instar larvae were used, and their blood, midgut, and whole body protein samples were prepared. The larvae's feet were cut, and blood was collected from the incision. The blood was then immediately stored at 4 °C to prevent oxidation and mixed with cold PBS buffer (stored at 4 °C and containing 1 mmol/L PMSF)[22] at 1:1 ratio and labeled as blood amylase crude extraction.[22] The blood samples were centrifuged at 9000*g* for 10 min at 4 °C before use. For midgut tissue samples, the larval midguts were dissected after being placed at −20 °C for a few minutes. Cooled PBS buffer (containing 1 mmol/L PMSF) was then added to the guts with a 1:1 ratio (w/v), and the mixture was homogenized on ice with a glass homogenizer. The homogenate solution was centrifuged at 9168*g* for 20 min at 4 °C; this supernatant was defined as midgut amylase crude extract and stored at −20 °C until further use. For whole body tissue samples, the fifth-instar larvae were homogenized as for the gut amylase crude preparation, after being placed at −20 °C to kill the larvae. The preparation for each sample was repeated three times and was conducted on ice or at 4 °C. The α-amylase activity was detected with an α-amylase assay kit (Beijing Kinghawk Pharmaceutical Co., Ltd, Beijing, China). The kit uses ethylidene *p*-nitrophenylmaltoheptaoside (EPS-G7) as substrate. Amylase hydrolyzes EPS-G7 to Et-Gx and Gy-pNP, and Gy-pNP is further hydrolyzed by a coupled enzyme to glucose and pNP, which can be quantitated at 405 nm. The amount of pNP formed is directly proportional to the amylase activity in the sample.

To analyze α-amylase activity, first, 5 μg of the recombinant α-amylase inhibitor protein was mixed with 10 μg of the amylase crude sample at room temperature. The plain PBS buffer was taken as the control. The reaction mixture was incubated at 37 °C for 10 min. Then the amylase activity was determined on the basis of absorbance measures made with a plate reader (Beijing Jinhao Pharmaceuticals Limited, Beijing, China) at a wavelength of 405 nm. The α-amylase activity (AMY) unit was calculated as

$$\mathrm{AMY\ (U/L)} = (\Delta A/\mathrm{min} \times V_t \times 1000)/(e \times V_s \times d)$$

where ΔA/min = the rate of absorbance change per minute, V_t = the total volume of reaction solution, e = 9.5 (molar absorptivity), V_s = the total volume of standard sample, d = the light path of the colorimetric cup, and 1000 is a variation factor.

Effects of α-Amylase Inhibitor Protein on *H. armigera* Larvae. The second-instar larvae were fed an artificial diet with a series of α-amylase inhibitor protein concentrations added. The α-amylase inhibitor protein concentrations were as follows (inhibitor protein/artificial diet): 5, 10, 20, 40, and 80 μg/g. The artificial diet containing PBS buffer was used as the blank treatment. The experiment was a completely randomized design with three replications. Twenty larvae were used for each replication. The average weight of a single larva in different treatment groups was recorded and calculated after 96 h. The samples of total soluble protein were collected, and the sample concentration was measured by the BCA method (Pierce, Rockford, IL, USA).

Statistical Analysis. All values were expressed as the mean ± standard error (SE) (n = 3), and the data were analyzed by ANOVA and the two-tailed Student's t test ($P < 0.05$).

RESULTS

Cloning and Sequence Analysis. A 360 bp putative gene encoded α-amylase inhibitor (short for SA-AI) was cloned. The inhibitor gene sequence was submitted to GenBank (ID FJ624422). The α-amylase inhibitor protein was predicted to be a 118 amino acid protein with a molecular mass of 12568 kDa, and it was predicted to contain a short signal peptide in the N-terminus (Figure 1). The amino acid sequence of the putative SA-AI was compared with other α-amylase inhibitors from *Streptomyces*. The results revealed that this protein contained conserved sites, including tryptophan−arginine−tyrosine (WRY) residues and cysteine residues (Figure 2). These conserved sites are considered to be important for the inhibitor activity of α-amylase inhibitors from *Streptomyces* and for the ability to bind to mammal amylases.[23]

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ATGAGGCGGTACATTCGTTCAACCATCACGGGGCTGACGGCAGGCTTGCTCGCCGTGTCG   60
 M  R  R  Y  I  R  S  T  I  T  G  L  T  A  G  L  L  A  V  S
TCCGGCGTGGCGGCGCAGGCGGCATCCCGCGAGGCAGAGGAAGGCTGGGACAGCCCGCCC  120
 S  G  V  A  A  Q  A  A  S  R  E  A  E  E  G  W  D  S  P  P
GCTTGCGTGCACATCAACTCGGACTGGCGCTACACGTTTGTCACCAACGACTGCTCGAGT  180
 A  C  V  H  I  N  S  D  W  R  Y  T  F  V  T  N  D  C  S  S
GCGTATGACGTGACTGTCGTCTATCGCGACGGAGCCAACGTCCCCTGCCGTGTCGCTTCC  240
 A  Y  D  V  T  V  V  Y  R  D  G  A  N  V  P  C  R  V  A  S
CCGGGTGACATCATCTCGTTCCCGGGCTATGGAACACAGGGCAATACGGTCCTCGGCGTC  300
 P  G  D  I  I  S  F  P  G  Y  G  T  Q  G  N  T  V  L  G  V
GCCCTGTGCGCGGTCGCAGGTCGGAAGGACGGCCGTTTGGAGGCGACTCCCCGCCTGTAG  360
 A  L  C  A  V  A  G  R  K  D  G  R  L  E  A  T  P  R  L  -
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Figure 1. Nucleotide and amino acid sequences of SA-AI. The signal peptide is underlined.

The sequence alignment results showed that the sequence for the SA-AI was similar to the sequences of Paim from *Streptomyces corchorusi* (BAA90724), Haim-2 from *Streptomyces griseosporeus* (AAA50324), T76 from *Streptomyces nitrosporeus* (AAC60452), and HOE407 from *Streptomyces tendae* (AAA26686).

α-Amylase Inhibitor Protein: Expression and Purification. The recombinant SA-AI accumulated in both the supernatant and the sediments of bacterial lysates, which suggested that the recombinant protein was mainly located in the periplasmic space of *E. coli* M15 cells (Figure 3). The apparent molecular weight of the recombinant SA-AI was 12500 kDa by SDS-PAGE detection, which was consistent with the predicted size (Figure 4).

Analysis of α-Amylase Activity and Effects of Inhibition. The amylase activities in the samples of larval midgut, blood, and whole body were compared to select the samples with higher amylase activity. The results showed that the larval blood sample had the highest amylase activity (66.205 U/L) compared to the midgut sample (62.627 U/L) and whole body sample (33.997 U/L). These results suggested that the larval blood sample was the best to be used for sample analysis. The results from this study indicated that the α-amylase inhibitor protein had effects on the amylase activity of *H. armigera*. The amylase activity was reduced by 22.22%

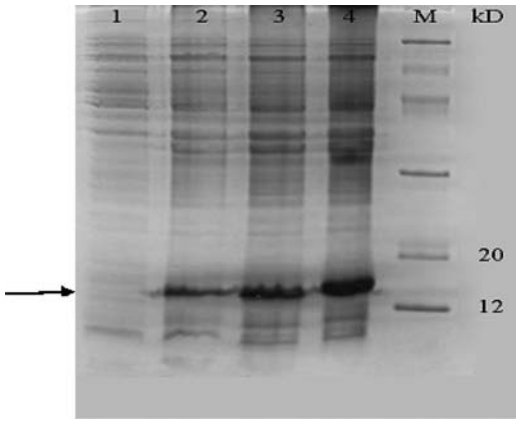

Figure 3. Recombinant SA-AI protein collected from *Escherichia coli* cells as shown by SDS-PAGE detection. The arrow indicates the position of recombinant SA-AI. Lanes: M, standard molecular mass marker; 1, uninduced M15 cells (containing the pQE-30-SA-AI vector); 2, M15 (containing the pQE-30-SA-AI vector) induced by IPTG; 3, bacterial lysate supernatant; 4, bacterial sediments.

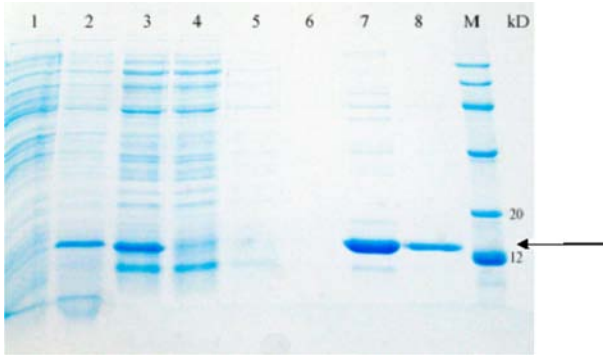

Figure 4. Purification of the recombinant protein. Lanes: 1, uninduced M15 cells (containing the pQE-30-SA-AI vector); 2, M15 (containing the pQE-30-SA-AI vector) induced by IPTG; 3, bacterial lysate supernatant; 4, eluent with bacterial lysate supernatant; 5–6, eluents with buffer; 7–8, eluents with 250 mM imidazole; M, standard molecular mass marker. The arrow indicates the purified recombinant protein.

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SA-AI     .QAASREAEEGWDSPPACVHINSDWRYTFVTNDCSSAYDV   39
PAIM      .QSDASEP......APACVVMYESWRYTTAANNCADTVSV   33
Haim-2    .DAGNRIA......APACVHFTADWRYTFVTNDCSIDYSV   33
T76      ATGAP.........APACVESFQSWRYTDVHNGCSETVSV   31
HOE407    .DTTVSEP......APSCVTLYQSWRYSQADNGCAQTVTV   33
Consensus                 p cv     wry    n c     v

SA-AI     TVVYRDGANVPCRVASPGDIISF.PGYGTQGNTVLGVALC   78
PAIM      SVAYQDGATGPCATLPPGAVTTVGEGYLGEHGHPDHLALC   73
Haim-2    TVAYGDGTDVPCRSANPGDILTF.PGYGTRGNEVLGAVLC   72
T76       TVEYTNGQWAPCVTIQPDGWATF.AGYGTNSNYVTAVKTC   70
HOE407    KVVYEDDTEGLCYAVAPGQITTVGDGYIGSHGHARYLARC   73
Consensus v y       c    p        gy           c

SA-AI     AVAG.RKDGRLEATPR                          93
PAIM      PSS.............                          76
Haim-2    ATDGSALPVDRERAVR                          88
T76       DPAATAS.........                          77
HOE407    L...............                          74
Consensus
```

Figure 2. Amino acid sequences of SA-AI and other α-amylase inhibitors from *Streptomyces avermitilis*.

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compared to the control when the *H. armigera* larval blood sample was treated with the α-amylase inhibitor protein.

Effects of α-Amylase Inhibitor Protein on *H. armigera* Larvae. The results showed that the α-amylase inhibitor protein had negative effects on larval growth. The average weight of larvae was reduced by treatment with the recombinant protein (Figure 5). With the α-amylase inhibitor

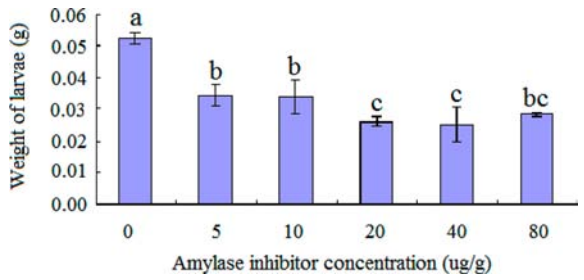

Figure 5. Effects of the α-amylase inhibitor protein on *Helicoverpa armigera* larval weight. The concentrations of the α-amylase inhibitor protein in the artificial diet were set at 0 (control), 5, 10, 20, 40, and 80 μg/g.

protein concentration at 5−80 μg/g (inhibitor/artificial diet), the larvae weight decreased as the amylase concentration increased compared to the control. The larvae fed an artificial diet with α-amylase inhibitor protein showed significant weight loss when the concentration of α-amylase inhibitor protein reached 20 μg/g and the average larval weight was reduced by approximately 50%.

The total soluble protein of larvae fed diets with different levels of α-amylase inhibitor protein added was also analyzed. The total soluble protein concentrations of larvae fed the artificial diet with different concentrations of added α-amylase inhibitor protein are shown in Figure 6. The results showed

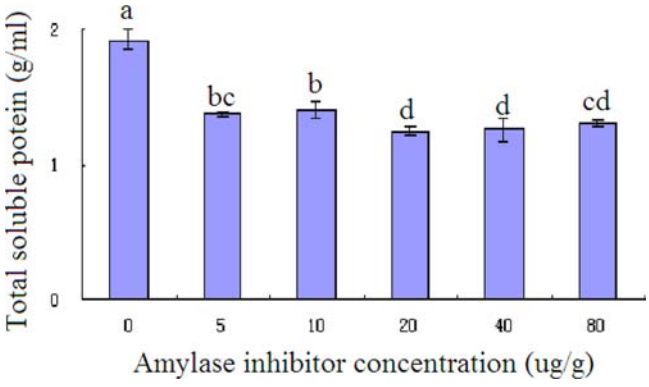

Figure 6. Effects of the α-amylase inhibitor protein on the total soluble protein of *Helicoverpa armigera* larvae. The concentrations of the α-amylase inhibitor protein in the artificial diet were set at 0 (control), 5, 10, 20, 40, and 80 μg/g.

that the α-amylase inhibitor protein reduced the total soluble protein concentration of larvae. With the α-amylase inhibitor concentration at 5 μg/g or above, the total soluble protein concentration of larvae was significantly reduced. As the α-amylase inhibitor increased, the total soluble protein concentration decreased. However, the larval growth was not linearly related to the total soluble protein concentration; this may be because the substrate for the protease inhibitor is limited at certain levels. With the α-amylase inhibitor concentration at 20 μg/g, the total soluble protein concentration of larvae was nearly half that of the control, which was consistent with the reduction of the larval weight. This result demonstrated that the α-amylase inhibitor protein had a negative effect on larval total soluble protein.

DISCUSSION

Homologous sequence alignment of α-amylase inhibitor proteins from *S. avermitilis* showed that the isolated protein was a member of *Streptomyces* protease inhibitors. The protein contained the conserved WRY amino acid site, which is the site where the inhibitor interacts with mammal amylases.[23,24] Previous studies have reported that this group of amylases has specific inhibition on mammal amylases. However, little information on these proteins acting against insect amylases has been reported. To the best of our knowledge, the present study is the first study to report the α-amylase inhibitor protein from *Streptomyces* acts against insect amylases and has adverse effects on insect growth and total soluble protein.

Because α-amylases play a central role in carbohydrate metabolism, organisms with a diet rich in starch depend on the effectiveness of their amylases for survival, which is certainly the case for insects that are serious agricultural pests because they consume plant organs rich in starch, such as seeds and roots.[23] The results from this study indicated that the new α-amylase inhibitor protein obtained was small in size, which can facilitate large-scale expression in *E. coli* cells and industrial production by fermentation, and the protein reduced the weight of *H. armigera* larvae. Therefore, the α-amylase inhibitor protein may be a candidate for a new bioinsecticide for controlling of insect pests. Future studies also can be conducted to evaluate the potential of the gene encoding this protein for use in genetic engineering of plants resistant to insect pests. However, because this α-amylase inhibitor protein family has an inhibition effect on animals, further studies need to be done to evaluate the effects of the protein on nontarget animals.

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Author Contributions

‖F.Z. and J.W. contributed equally to this paper.

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Notes

The authors declare no competing financial interest.

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