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A highly active esterase from *Lactobacillus helveticus* hydrolyzes chlorogenic acid in sunflower meal to prevent chlorogenic acid induced greening in sunflower protein isolates

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ABSTRACT

Chlorogenic acid (CGA) is an ester between caffeic and quinic acid. It is found in many foods and reacts with free amino groups in proteins at alkaline pH, leading to the formation of an undesirable green pigment in sunflower seed-derived ingredients. This paper presents the biochemical characterization and application of a highly active chlorogenic acid esterase from *Lactobacillus helveticus*. The enzyme is one of the most active CGA esterases known to date with a K_m of 0.090 mM and a k_{cat} of 82.1 s^{-1}. The CGA esterase is easily expressed recombinantly in *E. coli* in large yields and is stable over a wide range of pH and temperatures. We characterized CGA esterase's kinetic properties in sunflower meal and demonstrated that the enzyme completely hydrolyzes CGA in the meal. Finally, we showed that CGA esterase treatment of sunflower seed meal enables the production of pale brown sunflower protein isolates using alkaline extraction. This work will allow for more widespread use of sunflower-derived products in applications where neutrally-colored food products are desired.

1. Introduction

Chlorogenic acid (CGA) is a polyphenolic compound present in several foods, such as sunflower seeds and unroasted coffee beans (de Oliveira Filho & Egea, 2021; Santana-Galvez et al., 2017). While CGA is considered beneficial to human health for its antioxidant properties (Adeleke & Babalola, 2020; de Oliveira Filho & Egea, 2021), high concentrations of CGA are problematic in sunflower products because under alkaline conditions CGA reacts with free amines in proteins, forming a dark, aesthetically displeasing green trihydroxybenzacridine pigment (Fig. 1**A**; Wildermuth et al., 2016).

The majority (approx. 60 %) of sunflower seeds are used for sunflower oil production. The meal that remains after oil pressing is rich in nutrients such as protein and phenolics (Adeleke & Babalola, 2020). However, most sunflower meal (SFM) is currently either discarded, or used as fertilizer, or as animal fodder (de Oliveira Filho & Egea, 2021; Seiler et al., 2017). Using SFM as a source material to produce sunflower protein isolates (SPI) and sunflower flour would increase its utility. The main obstacle to utilizing SFM more widely is the high concentration of CGA in sunflower seeds. CGA constitutes up to 70 % of all phenolic compounds or approximately 1.4 % of dry weight (de Oliveira Filho & Egea, 2021; Santana-Galvez et al., 2017), causing the aforementioned greening reaction as CGA reacts with sunflower proteins under alkaline conditions (de Oliveira Filho & Egea, 2021; Pickardt et al., 2009; Wildermuth et al., 2016). This greening reaction means that lightly colored sunflower proteins must currently be made using acidic extraction instead of higher-yielding alkaline isoelectric precipitation methods (González-Pérez & Vereijken, 2007; Pickardt et al., 2009). Greening also

Abbreviations: CA, caffeic acid; CD, circular dichroism; CGA, Chlorogenic acid; DLS, dynamic light scattering; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; IPTG, Isopropyl β-d-1-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser desorption/ionization – time of flight; QA, quinic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SFM, sunflower meal; SPI, sunflower protein isolate.

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prevents the use of SFM for applications where high pH is used and neutral colors are preferred, such as for baking cookies, bread, and muffins (de Oliveira Filho & Egea, 2021).

Chlorogenic acid esterase (CGA esterase) is a hydrolase that breaks down CGA into caffeic and quinic acid (Fig. 1**A**). The enzyme features a typical α/β hydrolase fold and cleaves CGA using a Ser-His-Asp catalytic triad (Lai et al., 2011). CGA esterases are part of the large ferulic acid esterase family of enzymes (EC 3.1.1.73) that are characterized by their ability to hydrolyze alkyl chain esters of hydroxycinnamic acid derivatives. Although all ferulic acid esterases feature an α/β hydrolase fold, they are diverse in structure, substrate specificity, and active site architecture (Lai et al., 2011). CGA esterases have been discovered in both bacterial and fungal species, notably among the fungal genus *Aspergillus* (Benoit et al., 2007; Faulds et al., 2005) and the bacterial genus *Lactobacillus* (Lai et al., 2009; Song & Baik, 2017). Bacterial and fungal CGA esterases differ substantially from each other in terms of sequence and structure (Hermoso et al., 2004; Lai et al., 2011). Structural analysis using the DALI protein structure comparison server (Holm, 2020) indicates that bacterial CGA esterases are most similar to human monoglyceride lipases and bacterial peroxidases. The main structural feature characteristic of bacterial CGA esterases is an α/β insertion domain in proximity to the active site (Fig. 1**B**), which is absent in all known fungal counterparts (Goldstone et al., 2010; Lai et al., 2011). Furthermore, unlike fungal CGA esterases, the bacterial enzymes are not glycosylated and therefore easier to express recombinantly in bacteria (Benoit et al., 2006).

Fungal CGA esterases have received significant attention for their biotechnological potential. In addition to hydrolyzing CGA, CGA esterases are able to release hydrocinnamic acid derivatives from the cell wall of plants. Therefore, they are of great interest to the pulp and paper and biomass processing industries (Dilokpimol et al., 2016; Goldstone et al., 2010; Nieter et al., 2015; Nieter et al., 2016). Compared to fungal CGA esterases, the functional potential of bacterial CGA esterases remains unexplored. While some kinetic and enzymatic properties of CGA esterase from *L. helveticus* (Song & Baik, 2017), *L. gasseri*, *L. acidophilus* (Fritsch et al., 2017), *L. plantarum* (Esteban-Torres et al., 2015; Esteban-Torres et al., 2013), and *L. johnsonii* (Lai et al., 2009) were reported, bacterial CGA esterases have yet to be tested in food applications.

Since the CGA concentration in sunflower seeds is high, preventing CGA-induced greening in sunflower seed-derived products has been challenging. Several attempts have been reported in the literature, however, none are fully satisfactory. For example, it is possible to extract CGA using organic solvents such as methanol or obtain sunflower proteins by membrane filtration (Gonzalez-Perez et al., 2002; Pickardt et al., 2009; Wianowska & Gil, 2019). However, these approaches remove beneficial phenolics present in sunflower seeds and introduce the problems of solvent recovery, cost, and safety. Attempts to mitigate greening in SPI and solutions by altering redox conditions using cysteine

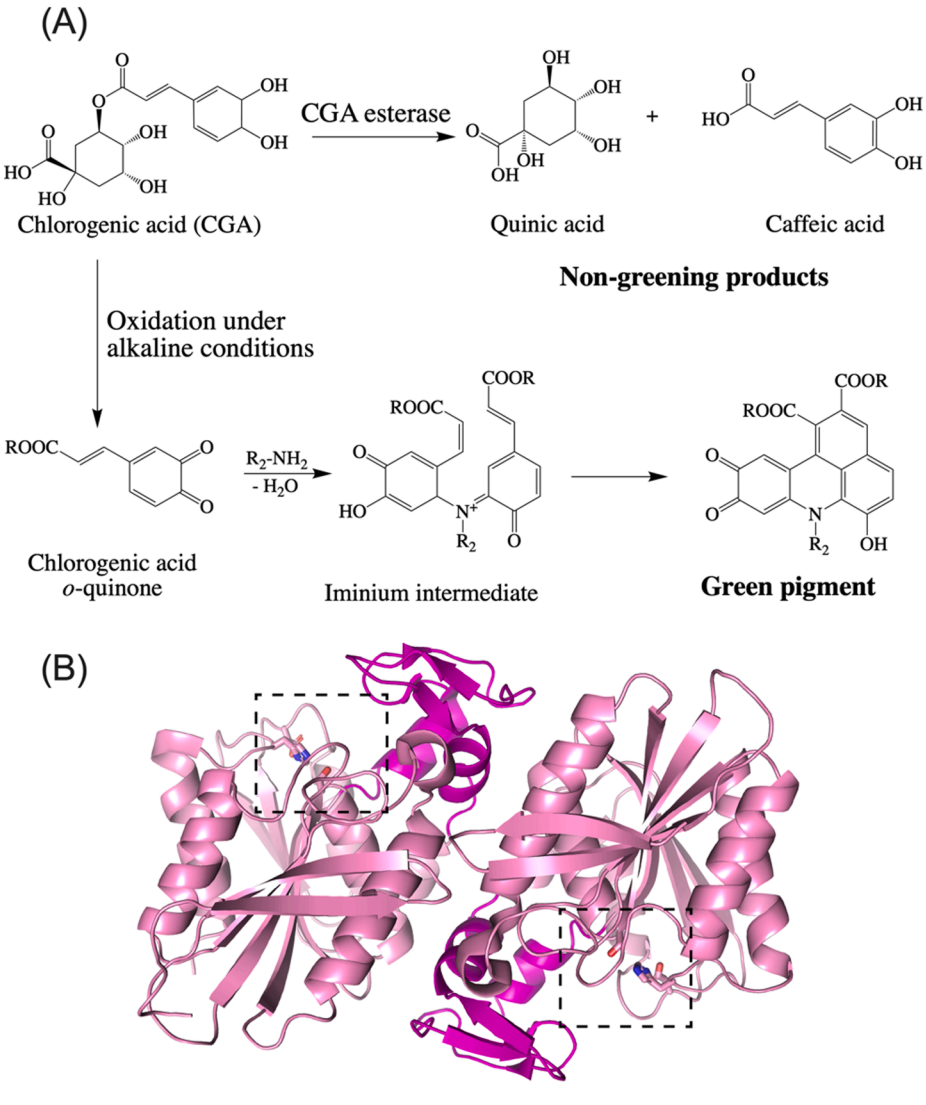

Fig. 1. (A) Proposed reaction mechanisms for CGA breakdown in sunflower-derived ingredients and CGA-induced greening. Greening arises when trihydroxybenzacridine derivatives form, as described in Bongartz *et al.* (2016). (B) Structure of CGA esterase (PDB ID: 3PF8) from *Lactobacillus johnsonii*. Dark pink strands demark the characteristic insertion domain, and the dashed boxes indicate the location of the catalytic triad (Ser-Asp-His) in the active site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

have been successful (Ishii et al., 2021; Liang & Were, 2020). However, this approach only works when cysteine levels are high (above 2.1 mM) and is not compatible with foods that carry a "clean label" claim. Finally, a study by Zhang, Liu, Hu & Yang, (2019) attempted to hydrolyze CGA and prevent greening using a fungal CGA esterase from *Aspergillus niger*. The study indicated that *A. niger* CGA esterase hydrolyzed CGA, however, there was no direct evidence of enzymatic greening prevention since the SFM was partially de-phenolized by organic solvent extraction and the experiment was conducted entirely at acidic pH, which in and of itself mitigates greening (Atonfack et al., 2019; Liang et al., 2018). In addition to the lack of evidence for greening prevention, CGA esterase's enzymatic and physical properties in food matrices are nearly uncharacterized.

Our research objective was to test the hypothesis that a *Lactobacillus* CGA esterase will hydrolyze CGA from SFM and prevent greening in SPI. We demonstrate that CGA esterase from *L. helveticus* is one of the best-performing CGA esterase known to date. We show that the enzyme is efficiently expressed in *E. coli*, is very stable and active under a wide range of conditions, and that CGA esterase breaks down CGA in SFM. Finally, we demonstrate that CGA esterase treatment enables production of light-brown sunflower-seed derived meal and protein powders.

2. Materials and methods

2.1. Reagents and materials

All reagents and HPLC consumables were purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Hampton, NH). Lyric Wild Bird Food sunflower seeds were purchased from Home Depot (California, USA).

2.2. Chlorogenic acid esterase cloning

The gene coding for CGA esterase was purchased from Genewiz (Cambridge, MA) and was codon-optimized for expression in *E. coli*. The CGA esterase gene was amplified using the following primers by PCR:

Forward: 5′ GCCCGCTAGCATGAGCCGCATTACCATTGAACGC.

Reverse: 5′ CCGCGAGCTCTTAAAACGCCGGTTTCAGAAACTGCG.

The forward and reverse primers have an Nhe1 and Sac1 restriction enzyme site, respectively, and were inserted into a pET28a plasmid by restriction digest cloning.

2.3. Chlorogenic acid esterase purification and expression

CGA esterase was expressed in *E. coli* (BL21) in 2.8 L Erlenmeyer flasks containing 1.0 L LB (Miller) broth and 30 mg/L kanamycin. Cells were grown for approximately 2–4 h at 37 °C in an orbital shaker set to 250 rpm until they reached an optical density (OD) at 600 nm of 0.6–1. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. The incubation temperature was then lowered to 18 °C and the expression continued overnight (16–20 h) with an orbital shaker speed of 200 rpm. Cells were then harvested by centrifugation at 5,000 rpm for 10 min and the pellet was transferred into Falcon tubes and centrifuged again at 5,000 rpm for 10 min. The supernatant was decanted and the pellet was stored at −20 °C until use.

Cells were lysed by microfluidization at 16,000 psi and the lysate was clarified by centrifugation at 12,500 rpm for 45 min at 4 °C. The supernatant containing CGA esterase was loaded onto a 5 mL Ni^{2+} column (Cytiva Life Sciences; column dimensions: 1.6 × 2.5 cm) pre-equilibrated with 50 mM Tris pH 8.0, 100 mM NaCl, and 20 mM imidazole. For elution of CGA esterase from the column, 50 mM Tris pH 8.0, 100 mM NaCl, 500 mM imidazole was applied using a linear gradient at a flow rate of 2 mL min^{-1}. SDS-PAGE was used to detect fractions containing CGA esterase and fractions containing the enzyme were pooled. This was followed by a two-step dialysis against a buffered solution containing 50 mM HEPES pH 8.0, 500 mM NaCl, and 10 % glycerol. Each dialysis step proceeded for at least 6 h. After dialysis, CGA esterase was concentrated using an Amicon stirred cell with a 10 kDa membrane to approximately 10 mL and further concentrated in a 10 kDa centrifugal filter to approximately 5 mL. Purification was completed using an S200 gel filtration column (Cytiva Life Sciences; column dimensions: 1.0 × 30.0 cm) pre-equilibrated with a buffered solution containing 50 mM HEPES pH 8.0, 500 mM NaCl, 10 % glycerol at flow rate of 0.5 mL min^{-1}. No more than 5 mg of protein were loaded on the column per run using a loading volume of 0.5 mL. Fractions containing CGA esterase were detected using SDS-PAGE and pooled. Enzyme concentration was calculated using an extinction coefficient of 14,900 M^{-1} cm^{-1} at 280 nm, which was predicted by Expasy Protparam based on the protein's Trp and Tyr content (Gasteiger et al., 2005).

2.4. Circular dichroism, dynamic light scattering and mass spectrometry

Circular dichroism (CD) spectra were taken on a Jasco J-1500CD spectrophotometer. CGA esterase (0.2 mg mL^{-1}) was buffered in a solution of approximately 5 mM HEPES, pH 8.0. Spectra were recorded at 4 °C using the following settings: 100 nm min^{-1} scan rate, 0.2 nm data pitch, 1.0 nm bandwidth, and 2 s integration time. Five acquisitions were averaged per experimental sample. To generate thermal denaturation curves, the CD signal at 222 nm was recorded as the temperature was gradually increased from 4 °C to 96 °C. The T_m was estimated by fitting a Boltzmann sigmoidal function to the denaturation curve. For CD spectra that were run at different pH, a concentrated CGA esterase stock solution was diluted to either 0.2 mg mL^{-1} or 0.1 mg mL^{-1} into solutions of 10 mM buffer at the pH indicated in the figure.

Dynamic light scattering (DLS) experiments were carried out using a Wyatt DynaPro Nanostar instrument set at 21 °C. Samples were prepared in 50 mM HEPES pH 8.0, 500 mM NaCl, 10 % glycerol that had been filtered twice using a 0.2 μm filter. Each CGA esterase measurement consisted of 10 scans with a 5 s acquisition time. The detector angle was set at 163.5° and the wavelength was equal to 532 nm.

The molar mass of CGA esterase was determined by MALDI-TOF mass spectrometry on intact protein using methods described previously (Medina et al., 2021).

2.5. Enzymatic assays to determine Michaelis-Menten parameters

Michaelis-Menten assays were conducted at 21 °C. CGA hydrolysis was monitored using an Agilent Technologies Cary 60 UV–vis spectrophotometer. Hydrolysis was observed by absorption spectroscopy since HPLC-based methods would have been too slow to monitor the rapid reaction. A stock solution of CGA was prepared by dissolving CGA in 50 mM HEPES pH 8.0. The CGA concentration was calculated using the Lambert-Beer law with an experimentally determined extinction coefficient of 17,194 M^{-1} cm^{-1} at 330 nm at pH 8.0. Subsequent dilutions of stock CGA were prepared to achieve final CGA concentrations of 0.02, 0.04, 0.06, 0.10, 0.20, and 0.40 mM CGA. The final CGA esterase concentration in all enzymatic assays was 10 nM unless noted otherwise. All samples used for enzymatic assays were buffered in 50 mM HEPES, pH 8.0. Absorption scans were taken at 0.05 or 0.1 min intervals for 2 min between wavelengths of 250 and 400 nm. For CGA concentrations below 0.1 mM, the pathlength was 1 cm. Above 0.1 mM CGA, the pathlength was 0.5 cm.

To measure CGA depletion (Δ[CGA]), **Eq. (1)** was used:

$$\Delta[CGA] = \frac{A - A_o}{\varepsilon_{CA} - \varepsilon_{CGA}} \quad (1)$$

A is the absorbance at any time point in the reaction, A_o is the initial absorbance, ε_{CA} and ε_{CGA} are the extinction coefficients at 340 nm of caffeic acid (CA) and CGA, which are 4.15 and 14.43 mM^{-1} cm^{-1}, respectively. To generate initial rates, |Δ[CGA]| was plotted against time. The change in absorbance during the first minute of the reaction

was fit to a linear curve, which is used to generate the reaction rate. The rate of CGA depletion is equivalent to that of CA formation. To test the effect of salt on enzyme kinetics, the same procedure was used, except that the experiment was run with 0.04 mM CGA buffered in 50 mM HEPES pH 8.0 with 500 mM NaCl.

2.6. Temperature-dependence measurements

A circulating water bath was used to run enzymatic reactions at the temperatures indicated in the text to maintain the temperature in the spectrophotometer within 1 °C of the target temperature. Buffered solution, CGA, and CGA esterase samples were pre-incubated for 10 min at the desired temperature before starting a measurement. All other instrument parameters were the same as for room temperature measurements described in section 2.5.

2.7. Sunflower meal and sunflower protein treatment with chlorogenic acid esterase

Sunflower seeds were ground using a KitchenAid coffee grinder in two 30 s increments and sieved through a 500 μm filter to make SFM. Two defatting methods were used: cold press and hexane extraction. To make cold pressed SFM, ground seeds were pressed in a Carver Hydraulic Unit Model 3912 3852–0 five times to a pressure of 9,000–10,000 psi. For hexane extracted SFM, fat was removed from ground seeds using a Soxhlet apparatus in 250 mL hexane for 18 h and subsequently dried using a Fischer Scientific Isotemp Oven Model 516G at 45 °C for 3 h. Cold pressed, hexane extracted, and undefatted SFM were then sieved through a 500 μm filter.

To make SPI, 12.5 g of cold pressed, hexane extracted, and undefatted SFM were buffered in 50 mM HEPES at pH 8.0. Samples were continuously stirred for about 10 min until all SFM was completely suspended. Then, 0.2 mg of CGA esterase per g of SFM was added. The final volume for all reactions was 125 mL. In untreated samples, a buffered solution of 50 mM HEPES, pH 8.0 was added instead of the enzyme solution. After 10 min, 0.1 M NaOH was added to each solution until pH reached 9.00 ± 0.03. Samples were left to sit for 30 min and then centrifuged at 5,000 rpm for 20 min at 4 °C. The supernatant was placed back into a 250 mL beaker and pH was lowered using 0.1 M HCl to reach an isoelectric pH of 5.00 ± 0.03 (González-Pérez & Vereijken, 2007). Samples were allowed to precipitate at 4 °C overnight (14–16 h). The supernatant was discarded, and the precipitate was lyophilized for 20 h. All experiments (controls and CGA esterase treated samples) were duplicated.

2.8. Chlorogenic acid esterase activity in the sunflower meal supernatant and pellet

SFM (1 g) that was cold pressed, hexane extracted, or undefatted was buffered in 10 mL of 50 mM HEPES, pH 8.0. CGA esterase was added to a final concentration of 0.756 μM (0.02 mg mL^{-1}). Untreated samples received a solution of 50 mM HEPES, pH 8.0 instead of CGA esterase. Samples treated with CGA esterase were allowed to sit for 10 min and subsequently centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through a 0.2 μm filter and loaded onto an HPLC while the pellet was lyophilized for 20 h. After lyophilization, 10 mL of 70 % ethanol was then added to the dried pellet for 1 h to extract CGA. The pellet was subsequently centrifuged at 10,000 rpm for 10 min. The supernatant was then filtered through a 0.2 μm filter and loaded onto an HPLC.

2.9. Chlorogenic acid esterase kinetics in sunflower meal

To determine the Michaelis-Menten parameters of CGA esterase in undefatted SFM, SFM suspensions were made at following concentrations: 1.0, 2.5, 10.0, 30.0, and 100.0 mg mL^{-1}. All samples were made to a final volume of 10 mL in a solution containing 50 mM HEPES, pH 8.0. Treated samples contained a final enzyme concentration of 10 nM. Reactions were stopped by acidifying the solution by adding 120 μL of 37 % HCl at regular intervals for 600 s. Samples were subsequently centrifuged at 10,000 rpm for 10 min. Control samples confirmed that stopping the reaction by acid quenching does not induce CGA hydrolysis. The supernatant was then filtered using a 0.2 μm filter and loaded onto an HPLC, as described in section 2.10. The initial amount of CGA that was present prior to adding enzyme was measured using HPLC.

2.10. HPLC quantification of CGA and CA in sunflower meal and protein isolates

All solutions were diluted tenfold in 0.1 % formic acid in HPLC-grade water before analysis. Quantification of CGA and CA concentration was performed using an Agilent 1260 series HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump, an automated sample injector, a diode array detector, and a 4.6 mm × 150 mm Luna C18(2) column with a 5 μm particle size and 100 Å pore size (Phenomenex, Torrance, CA, USA). The injection volume was 20 μL. To elute analytes, mobile phases composed of 0.1 % formic acid in HPLC-grade water (A) and 0.1 % formic acid in HPLC-grade acetonitrile (B) were used in a linear gradient. The gradient is as follows: 0 to 13 % B in 1 min, up to 27 % B at 8 min, up to 100 % B at 8.5 min, isocratic at 100 % B to 9.5 min, 100 % B returned to 0 % B at 9.6 min with 3 min post-time. The concentrations of CGA and CA were calculated from peak integration data based on 10-point standard curve that spanned a concentration range of 0 to 0.1 mg mL^{-1}.

2.11. Colorimetry of sunflower protein powders

A Hunter colorimeter (Konica Minolta, Inc. Tokyo, Japan, model: CM-2500D) was used to measure color changes in CIE L* a* b* in the protein powders after calibration using a white plate. Protein powders were put into glass containers and placed on a white background before taking measurements.

3. Results

3.1. Choice of source organism for the chlorogenic acid esterase

To date, putative CGA esterases have been found in only a small number of bacterial species, mostly among the genus *Lactobacillus* (Fritsch et al., 2017; Lai et al., 2011; Song & Baik, 2017). The amino acid sequences of *Lactobacillus* CGA esterases are conserved (Fig. S1**A**) and their protein structures are predicted to be similar (Fig. S1**B**). In a comparative study, the activity of several CGA esterases was assessed by measuring the hydrolysis of the model substrate *p*-nitrophenyl acetate (Fritsch et al., 2017). This study revealed that hydrolysis activity was similar across CGA esterases, differing only about 50 % between species. Since no CGA esterase exhibited higher activity than the rest, we focused on a CGA esterase with good thermostability. CGA esterase from *L. helveticus* was selected since it was reported to be both thermostable and among the more active CGA esterases.

3.2. Expression, purification, and physical characterization of chlorogenic acid esterase from L. helveticus

CGA esterase from *L. helveticus* (Genbank ID: WP_025283955.1, Uniprot ID: U6F2K7) was cloned into a pET28a expression plasmid, heterologously expressed in *Escherichia coli* as a His-tagged fusion protein and purified in two steps by affinity and gel filtration chromatography. CGA esterase eluted in three peaks from the gel filtration column. The elution volume of the main peak indicated that the protein had a size of approximately 60 kDa, which is consistent with the expected dimeric state of CGA esterase (Fig. 2**A and S2**). After the gel filtration

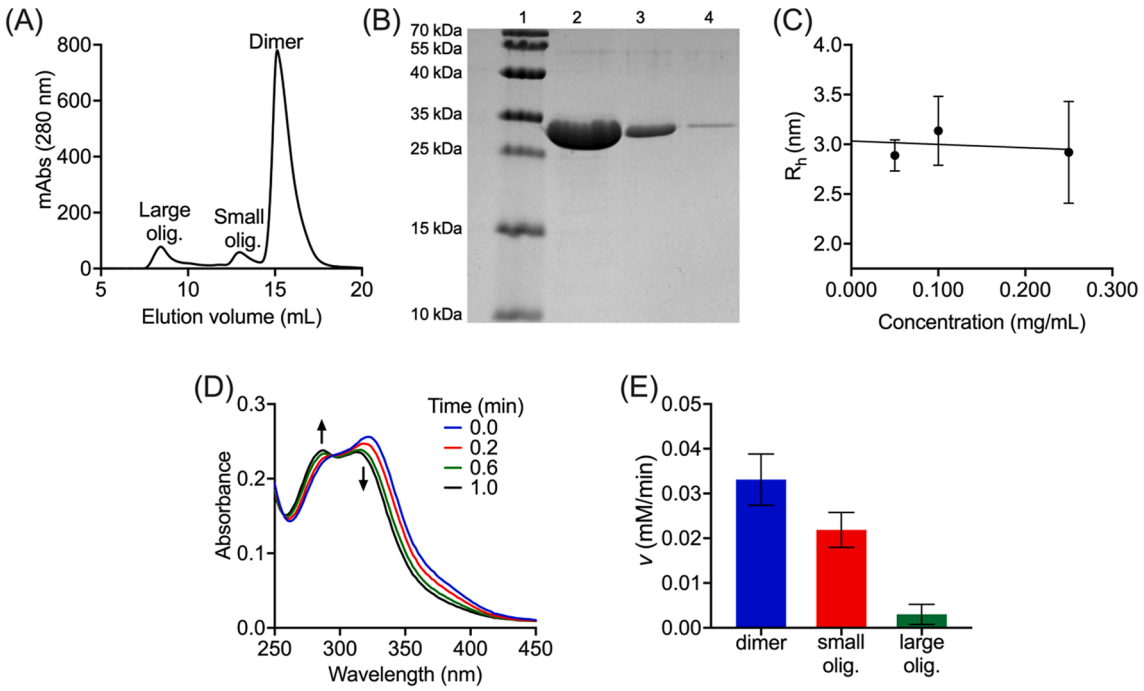

Fig. 2. Purification and physical properties of CGA esterase. (A) Gel filtration chromatogram of CGA esterase indicating that it elutes in three different aggregation states. (B) SDS-PAGE of CGA esterase following gel filtration, where lane 1 is the protein ladder and lanes 2–4 contain 0.069, 0.0069, and 0.00069 mg of CGA esterase, respectively. (C) CGA esterase radius measured by DLS indicating that the concentration independent radius is 3.03 nm, consistent with a ca. 60 kDa protein. (D) Absorption spectra of the hydrolysis of 0.02 mM CGA by CGA esterase over time. Arrows indicate changes in absorbance relating to decreasing CGA and increasing CA concentrations. (E) Rate of 0.04 mM CGA breakdown by the CGA esterase in its three aggregation states, demonstrating that the dimer is the active form of the enzyme.

step, there were no detectable impurities (Fig. 2**B**). The typical yield of purified CGA esterase after gel filtration was 20 mg of protein per L of growth media.

The oligomeric state of the main gel filtration peak was confirmed by DLS, which revealed a concentration independent radius of 3.03 ± 0.2 nm (Fig. 2**C**). This corresponds to the expected radius of a 60 kDa globular protein. The two minor peaks correspond to a trimer or tetramer (henceforth referred to as small oligomer) and a large oligomer, respectively. Mass spectrometry (**Fig. S3**) confirmed that CGA esterase had the expected molecular mass of 30 kDa per protomer. In addition, mass spectrometry revealed partial gluconylation of the protein, a common *N*-terminal posttranslational modification of proteins that are recombinantly expressed in *E. coli* (Aon et al., 2008).

The functional properties of the small and large oligomer of CGA esterase were unclear. To determine the relative activity of the different oligomers, we analyzed the rates of CGA hydrolysis into caffeic and quinic acid by absorption spectroscopy (Fig. 2**D and S4**). The dimer had the largest rate of CGA hydrolysis, indicating that it represents the active form of the enzyme (Fig. 2**E**). The rate of hydrolysis of the small oligomer peak was about half of that of the dimer and the large oligomer was inactive. The secondary structures of the dimer, small and large oligomers of CGA esterase were analyzed by CD spectroscopy. CGA esterase is expected to consist of a mix of α-helices and β-sheets (**Fig. S1**). While the spectrum of the dimer was consistent with a mixed α/β-domain protein, the signals of the oligomers were weaker, which indicates partial protein unfolding (Fig. 3**A and S5**). Thus, the lower activity of oligomers was likely due to a loss in secondary structure. Since the oligomers represent only a minor component of the purified protein, they were not used for further experiments. Follow-up gel filtration experiments demonstrated that the dimeric enzyme does not aggregate after purification (**Fig. S2**) and stays stable at elevated temperatures for hours (**Fig. S5**), meaning that CGA esterase will not lose activity over time by unfolding or oligomerizing.

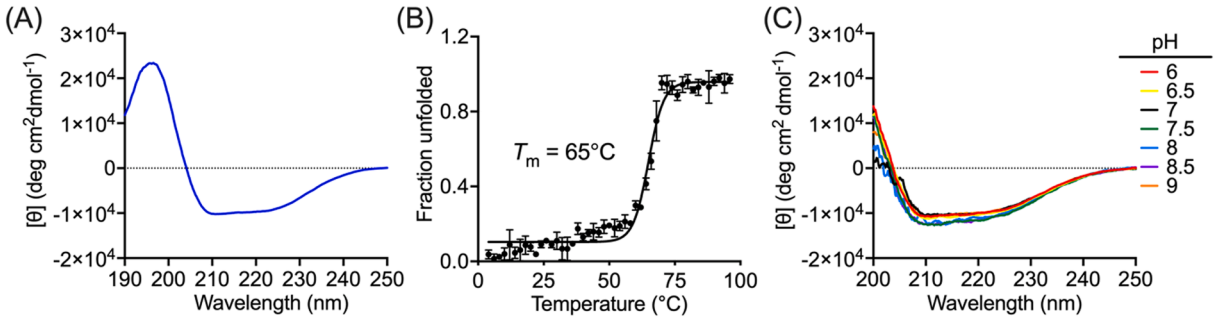

Fig. 3. (A) CD spectrum of CGA esterase at room temperature (21 °C), suggesting a mixture of α-helices and β-sheets. (B) Thermal denaturation curve of CGA esterase. (C) Room temperature (21 °C) CD spectra of CGA esterase at pH 6.0–9.0, indicating that the protein is stable over a wide range of pH.

3.3. Thermal and pH stability of chlorogenic acid esterase

We determined CGA esterase's structural stability by CD spectroscopy, which demonstrated that the protein is thermostable with a T_m of 65 °C (Fig. 3**B**). Once denatured, the protein does not refold when cooled back to room temperature (Fig. S5**B**). CGA esterase was also stable over a range of pH, as CD spectra were identical between pH 6.0 and 9.0 (Fig. 3**C**).

3.4. Kinetic characterization of chlorogenic acid esterase

The extent to which buffer composition influences protein activity was determined since protein yields were highest when purification was carried out in a buffer containing 500 mM salt. As shown in Fig. S6**A**, reactions carried out in 50 mM HEPES, pH 8.0 had higher activity than in the purification buffer, suggesting that kinetic experiments could be carried out in absence of salt. We next determined that no CGA hydrolysis occurred without enzyme and that the rate of CGA hydrolysis was directly proportional to the amount of enzyme (Fig. S6**B and S6C**). This indicates that all CGA hydrolysis was entirely an effect of enzymatic catalysis. Finally, we determined how pH affects enzyme activity, finding that the enzyme is active between pH 6.0 and 8.0 (Fig. S6D**D**).

The Michaelis-Menten parameters for CGA esterases are shown in Fig. 4 and Table 1. The K_m is 0.090 ± 0.038 mM, V_{max} is 170.2 ± 32.3 mM $min^{-1}mg^{-1}$, and k_{cat} is 82.1 ± 15.6 s^{-1}. The K_m is comparable to reported values for CGA binding to *L. johnsonii* CGA esterase and the previously reported value for *L. helveticus* CGA esterase (Lai et al., 2009; Song & Baik, 2017). In contrast, V_{max} is 300-fold higher than previously reported for *L. helveticus* CGA esterase (Song & Baik, 2017), and 3-times greater than that for *L. johnsonii* CGA esterase (Lai et al., 2009). We would like to note that the V_{max} and k_{cat} values reported by Song & Baik are inconsistent with each other since their reported k_{cat} value is similar to ours (Table 1). Our measurements represent independent averages from CGA esterase that were generated from multiple different growths and purifications. Furthermore, the accuracy of the absorbance-based CGA hydrolysis assays was confirmed using an HPLC-based measurement (**Fig. S7**). We are therefore confident that the high V_{max} for *L. helveticus* CGA esterase is accurate.

Since enzyme activity is often temperature-dependent, we determined to what extent CGA esterase activity changes with temperature. CGA esterase activity increases only weakly at elevated temperatures, as shown in **Fig. 4 and S8**. The Michaelis-Menten parameters at room temperature and 40 °C are very similar (Fig. 4). Further measurements of the rate of CGA hydrolysis in 10 °C temperature increments between 20 °C and 50 °C confirmed that enzymatic activity only increases slightly with elevated temperatures (**Fig. S8**). The small temperature-dependence of *L. helveticus* CGA esterase is similar to that of CGA esterases from the bacteria *L. gasseri* and *Bif. animalis* with the model substrate *p*-nitrophenyl acetate, which had a very weak temperature-dependence between 20 °C and 40 °C (Fritsch et al., 2017).

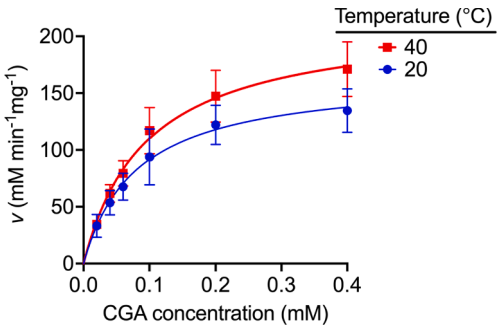

Fig. 4. Michaelis-Menten analysis of CGA esterase at 20 °C and 40 °C. Data is based on four independent triplicate averages. At 20 °C, K_m is 0.090 ± 0.038 mM, V_{max} is 170.2 ± 32.3 mM min^{-1} mg^{-1}. At 40 °C, K_m = 0.096 ± 0.006 mM and V_{max} is 215.4 ± 31.7 mM min^{-1} mg^{-1}.

3.5. Enzymatic CGA hydrolysis in sunflower meal

After demonstrating the effectiveness of CGA esterase at cleaving CGA, we tested if the enzyme could hydrolyze CGA in SFM. Sunflower seeds have a high lipid content of about 50–60 % w/w (de Oliveira Filho & Egea, 2021). Since CGA esterase has structural similarity to monoglyceride lipases, we were concerned that lipids interfere with CGA binding to the enzyme. We, therefore, compared the enzyme's performance in seeds that were undefatted to those that were defatted using two different methods: cold pressing and hexane extraction.

CGA hydrolysis in the meal was initiated by adding CGA esterase to SFM that was suspended in a buffered solution. To determine whether hydrolysis was taking place in both CGA that diffused into the aqueous phase and CGA that remained stuck in the suspended meal particles, CGA hydrolysis was measured for both. As shown in Fig. 5**, S9**, and Table 2, CGA esterase rapidly cleaved CGA in each of the three different types of samples, regardless of lipid content. Cleavage was complete (greater than 99 %) in defatted samples and ca. 96 % complete in undefatted samples. Furthermore, CGA was hydrolyzed in both the aqueous solution and suspended meal. Additional experiments described in Pepra-Ameyaw *et. al.* (manuscript under review) demonstrate that CGA esterase also hydrolyzes CGA in dry meal.

3.6. Kinetics of CGA hydrolysis in sunflower meal

We next were interested in determining to what extent the kinetics of CGA hydrolysis in SFM differed from the kinetics with pure CGA in buffer. To do so, the concentration of SFM, and thus of CGA, was varied to obtain the enzyme's K_m and V_{max} when operating in the meal. As shown in Fig. 6 **and S10**, enzyme activity increased hyperbolically with meal concentration, as expected. The K_m and V_{max}, listed in Fig. 6, were both lower than in buffered solution. Overall, the kinetics data indicates that maximal CGA hydrolysis activity is achieved when the concentration of the meal is approximately 100 mg mL^{-1}.

3.7. Production of sunflower protein from enzymatically treated meal

After demonstrating that CGA esterase hydrolyzes CGA in SFM, we investigated if enzymatic treatment improves the color properties of SFM-derived protein isolates. Protein isolates were produced from meal using alkaline extraction since this method provides higher protein yield compared to acidic extraction and is more widely used in isolating plant protein (Sathe et al., 2018). Experiments were run with undefatted meal, and defatted meal that was either cold pressed or hexane extracted. As shown in Table 3 and **Fig. S11**, CGA hydrolysis was complete in samples that were treated with CGA esterase, whereas CGA was present in samples that did not receive enzyme.

Finally, we determined that CGA esterase improves the color of SPI. As shown in Fig. 7 **and S12**, all untreated protein isolates displayed a characteristic deep green color, regardless of fat content. In stark contrast, all enzymatically treated samples were colored a pale shade of brown. CIE L*a*b* analysis results, shown in Table 4, indicate the enzymatic treatment significantly lowered the greening index (more positive value for a*) and increased lightness (higher L* value). Although all treatments provided complete greening prevention, there was a slight difference in color between the protein isolates that were cold pressed and solvent extracted. The color difference is in the brown range of the spectrum and likely due to Maillard reactions or phenol oxidation. Taken together, our data strongly suggests that enzymatic treatment of SFM completely prevents greening during alkaline protein isolation.

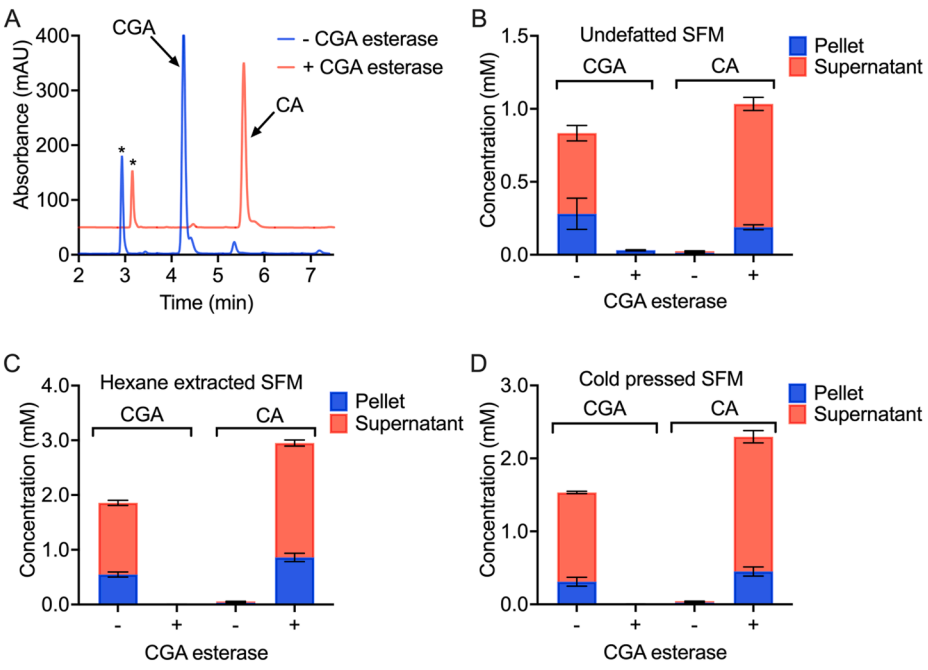

Fig. 5. (A) Representative HPLC chromatograms from SFM treated with CGA esterase (+) and untreated SFM (-). CGA and CA were detected at 320 nm. The retention times of CGA and CA are 4.3 min and 5.4 min, respectively. Peaks marked with an asterisk are other compounds found in the meal that are neither CGA nor CA. (B) Bar graph comparing CGA hydrolysis (formation of CA and depletion of CGA) in the pellet and supernatant in undefatted SFM, (C) hexane extracted SFM, and (D) cold pressed SFM. CGA breakdown is essentially complete in all three conditions.

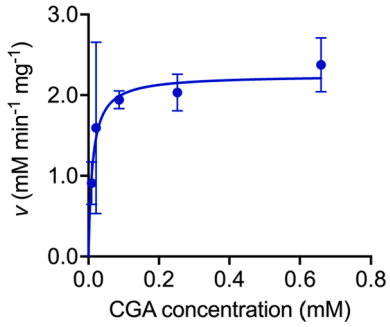

Fig. 6. Michaelis-Menten data for CGA esterase kinetics in SFM supernatant at pH 8.0 and 20 °C. Averaged data is processed from a minimum of three replicates. The K_m = 0.020 mM (95 % CI = 0.003 mM to 0.03 mM) and V_{max} = 2.716 mM min^{-1} mg^{-1} (95 % CI = 1.805 mM min^{-1} mg^{-1} to 2.746 mM min^{-1} mg^{-1}).

Table 1
Comparison of enzyme kinetics values for CGA esterases from fungal and bacterial sources toward the substrate CGA.

Source	K_m (mM)	V_{max} (mM min^{-1} mg^{-1})	k_{cat} (s^{-1})	Citation
A. niger	0.002	0.0228	0.5	Zhang et. al., 2019
A. niger	0.007	0.0150	1.9	Benoit et al., 2007
L. johnsonii	0.053	n/a	28.1	Lai et al., 2009
L. helveticus	0.153	0.5596	326.3*	Song & Baik, 2017
L. helveticus	0.090	170.2	82.1	This study

* k_{cat} and V_{max} values in Song & Baik (2017) are inconsistent with each other.

Table 2
Chlorogenic and caffeic acid content in the pellet and supernatant of CGA esterase treated and untreated sunflower meal powders.

Lipid extraction technique	CGA esterase	Pellet		Supernatant	
		mg CGA/g meal	mg CA/g meal	mg CGA/g meal	mg CA/g meal
Undefatted	–	0.99 ± 0.38	0.03 ± 0.00	1.96 ± 0.19	0.02 ± 0.00
	+	0.11 ± 0.01	0.34 ± 0.03	0.00 ± 0.00	1.52 ± 0.08
Hexane extracted	–	1.94 ± 0.16	0.06 ± 0.00	4.64 ± 0.16	0.04 ± 0.00
	+	0.03 ± 0.00	1.55 ± 0.14	0.00 ± 0.00	3.77 ± 0.10
Cold pressed	–	1.10 ± 0.22	0.04 ± 0.01	4.33 ± 0.06	0.03 ± 0.00
	+	0.02 ± 0.00	0.81 ± 0.11	0.00 ± 0.00	3.33 ± 0.15

– means that no enzyme and + means that enzyme is present.

Table 3
Total CGA and CA content in treated and untreated samples of sunflower protein isolates.

Lipid extraction technique	CGA esterase	mg CGA/g protein	mg CA/g protein
Undefatted	–	2.60 ± 0.17	0.08 ± 0.07
	+	0.00 ± 0.00	1.11 ± 0.38
Hexane extracted	–	3.90 ± 0.81	0.70 ± 0.19
	+	0.00 ± 0.00	3.38 ± 0.25
Cold pressed	–	4.23 ± 0.25	0.20 ± 0.04
	+	0.00 ± 0.00	3.62 ± 0.05

– means that no enzyme and + means that enzyme is present.

4. Discussion

Here, we characterized a thermostable CGA esterase from *L. helveticus* that was very active against CGA and completely hydrolyzed CGA in SFM. We further showed that CGA esterase treatment of SFM allows the production of SPI that are pale brown in color instead of green.

Prior to this work, there had been several reports on the biochemical and structural properties of bacterial CGA esterases (Fritsch et al., 2017; Lai et al., 2009; Song & Baik, 2017; Wang et al., 2004). Our biochemical characterization of *L. helveticus* CGA esterase largely agrees with previous work. The protein's secondary structure, thermostability, and the K_m towards CGA are similar to those of other *Lactobacillus* CGA esterases. There are, however, some key areas where our results differ from

Fig. 7. Protein isolates made from treating three types of sunflower meal.

Table 4
L*a*b* values of protein isolates made from treatment of undefatted and defatted (hexane extracted and cold pressed) samples treated with CGA esterase.

Fat extraction technique	CGA esterase	L*	a*	b*
Undefatted	–	24.31 ± 4.19	−7.88 ± 0.43	2.99 ± 0.14
	+	43.05 ± 5.02	1.18 ± 1.02	8.14 ± 0.96
Hexane extracted	–	35.02 ± 8.97	−4.57 ± 2.20	2.02 ± 2.34
	+	34.28 ± 3.00	−0.48 ± 0.21	6.70 ± 2.17
Cold pressed	–	24.61 ± 0.91	−7.87 ± 0.45	3.55 ± 0.21
	+	39.86 ± 9.48	−0.61 ± 0.21	6.11 ± 2.67

– means that no enzyme and + means that enzyme is present.

previous reports. We purified *L. helveticus* CGA esterase as a dimer. This agrees with the oligomeric state found in the *L. johnsonii* CGA esterase crystal structure (Lai et al., 2011), but not with a previous report on *L. helveticus* CGA esterase that stated the enzyme was a monomer (Song & Baik, 2017). There is a large surface area between protomers in the CGA esterase crystal structure, which is consistent with our data that indicate the dimer remains stable over time and over a wide concentration range (Fig. 2). Together with our results that demonstrate that higher oligomeric states of CGA esterase are less active (Fig. 2), we conclude that the dimer is the native and functional form of the enzyme.

We did not observe a strong temperature dependence of the activity for CGA esterase between 20°C and 50°C (**Fig. S8**), which makes *L. helveticus* CGA esterase similar to *Bif. animalis* and *L. gasseri* CGA esterase, but different from other *Lactobacillus* CGA esterases (Fritsch et al., 2017). Thus, the temperature-dependence of the CGA hydrolysis reaction appears to differ based on the bacterial origin of the enzyme. The structural and mechanistic basis for this difference is unclear since bacterial CGA esterases are similar in terms of amino acid sequence and predicted structure. The varied temperature-dependence of bacterial CGA esterases merits further investigation since it may point towards hidden steps in the reaction mechanism, such as a conformational change in the enzyme, that have different temperature-dependencies depending on the enzyme.

The most significant difference between our work and previous reports is the unexpectedly high V_{max}. The reason for the large V_{max} is unclear. That said, *L. helveticus* CGA esterase's k_{cat} of 82.1 s^{-1} lies within the reported range of structurally related α/β hydrolases such as hormone-sensitive lipase family carboxylesterases (Chahinian et al., 2005) and bile salt hydrolases (Kumar et al., 2006) that cleave substrates that are similar in size to CGA. It is possible that the lower enzyme activity reported previously by Song & Baik (2017) for *L. helveticus* CGA esterase may be traced to differences in the enzyme's oligomeric state since the authors conducted their experiments with a monomeric, and thus possibly less active, form of the enzyme.

When compared to fungal CGA esterases, *L. helveticus* CGA esterase offers advantages. First, it is much more active. Fungal esterases from *A. niger* have a K_m in the low μM range and a V_{max} of 0.02 mM min^{-1} mg^{-1} (Asther et al., 2005; Benoit et al., 2007; Zhang et al., 2019), which is approximately 8,500-times lower than *L. helveticus* CGA esterase. Since CGA hydrolysis in SFM is not limited by substrate binding, the higher V_{max} for the bacterial enzyme suggests it will be more effective at hydrolyzing CGA. Second, *L. helveticus* CGA esterase maintains better thermostability than its fungal counterparts. *A. niger* CGA esterase is rapidly inactivated at temperatures above 40°C (Nieter et al., 2015; Zhang et al., 2019), whereas the *L. helveticus* enzyme remains fully folded at 40°C over time and active until at least 50°C (Fig. S5**B and S8**). Third, the yield of recombinantly expressed *L. helveticus* CGA esterase is approximately 20 mg of protein per L of growth medium which far exceeds that of *A. niger* CGA esterase, which is produced in the native host with yields of < 1 mg/L of growth medium (Asther et al., 2005; Nieter et al., 2015).

After characterizing the biochemical properties of *L. helveticus* CGA esterase, we determined that it cleaved CGA in SFM very effectively, achieving close to 100 % conversion of CGA into caffeic and quinic acid in 10 min. CGA esterase hydrolyzes CGA in aqueous solution and CGA that remains trapped within the meal (Fig. 5). To the best of our knowledge, this observation is the first instance that CGA esterase activity was directly demonstrated in both aqueous and solid media. Furthermore, CGA esterase hydrolyzes CGA to near completion in undefatted meal. This suggests that lipids do not significantly inhibit CGA esterase.

The kinetics of CGA hydrolysis in SFM differ from that in buffer alone. The apparent Michaelis-Menten parameters, K_m and V_{max}, were 0.020 mM and 2.716 mM min^{-1} mg^{-1}, respectively, indicating a 4.5-fold lower K_m and 60-fold lower V_{max} compared to CGA in a simple buffered solution. Kinetic analysis of enzymes in complex food mixtures is rarely done (Capuano et al., 2018). The decline in K_m and V_{max} is characteristic of an uncompetitive inhibition process. Sunflower seeds

are known to contain potent peptide inhibitors that act on trypsin proteases (de Veer et al., 2021). Since trypsin proteases have a similar active site and mechanism as CGA esterases, peptide inhibitors may lower CGA esterase activity in the meal.

While the presence of an inhibitor may explain the shifts in K_m and V_{max}, additional factors are likely at play. CGA hydrolysis in meal was conducted in a suspension. At the highest concentrations, the meal takes up approximately 10 % of the volume fraction and the suspension is thick. There is an exponential relationship between particle concentration (*i.e.* volume fraction) and viscosity (Metzner, 1985). It is likely that the decline in V_{max} is at least, in part, due to slower diffusion in a viscous environment. Similarly, enzyme diffusion through the meal particles, which have a diameter of approximately 500 μm, is likely slower than in dilute solvent solutions.

Finally, molecular crowding effects by the meal likely influence enzyme activity (Anand et al., 2019; Capuano et al., 2018) since crowding can influence the mobility of both enzyme and substrate, leading to slower reaction rates as described in several enzymatic systems (Anand et al., 2019; Ma & Nussinov, 2013). The crowding agent can also affect protein folding equilibria and aggregation states (Minton, 2000; Zhou, 2013). Since oligomeric CGA esterase is less active than the dimeric enzyme, crowding-induced oligomerization may explain the diminished activity of CGA esterase in the meal. To gain insights on the effects of molecular crowding on CGA esterase mobility, we plan on pursuing fluorescence microscopy experiments that are based on methods for tracking trypsin diffusion in complex food matrices (Rakhshi et al., 2022; Silva et al., 2013).

Despite its lower enzymatic activity in meal compared to buffered solution, CGA esterase is still effective at removing CGA in SFM. Its *in situ* activity is similar to lipases and xylanases, two hydrolases that are used in commercial breadmaking. CGA esterase was used at a concentration of 200 ppm (0.2 mg of enzyme per gram of SFM) to break down CGA in meal. This level is equivalent to a xylanase used at 200 ppm (Maat & Martinus, 1990) and similar to widely used lipases, such as Palatase 20,000, which are used at 100–300 ppm (Olesen, Si, & Donelyan, 2000; Sato, Sato & Nagashima, 1991). To demonstrate a potential application of CGA esterase, we produced pale brown SPI that were generated through an alkaline extraction process (Fig. 7). The method represents an improvement over existing methods of making SPI since it allows SPI to be made using the standard, high-yielding alkali process that is used for other seed-derived protein isolates (Sathe et al., 2018) without the need to de-phenolize SFM first. Moreover, enzymatic CGA removal was achieved within ten minutes using a one-step process that is quicker than existing multi-step extraction and filtration methods (Jia et al., 2021; Malik & Saini, 2017; Pickardt et al., 2009). Enzymatic CGA breakdown may, furthermore, yield environmental and safety benefits since it avoids the use of volatile organic solvents and acids for CGA extraction. We expect that making protein isolates from sunflower meal that is left over as a byproduct of sunflower oil pressing will be particularly useful. The meal is currently considered a low-value product that is either discarded or used as animal fodder. CGA esterase treatment will make the conversion of SFM into protein powders economically appealing by providing a process to make affordable and nutritious sunflower seed-derived protein powders.

Data Availability.

All data is contained in the article and supporting information.

CRediT authorship contribution statement

Christine Lo Verde: Conceptualization, Investigation, Supervision, Methodology, Writing – original draft. **Nana Baah Pepra-Ameyaw:** Conceptualization, Investigation, Supervision, Methodology, Writing – original draft. **Charles T. Drucker:** Investigation, Methodology, Writing – review & editing. **Tracie L.S. Okumura:** Investigation, Methodology, Writing – review & editing. **Katherine A. Lyon:** Investigation, Methodology, Writing – review & editing. **Julia C. Muniz:** Investigation, Methodology, Writing – review & editing. **Chloe S. Sermet:** Investigation, Methodology, Writing – review & editing. **Lilian Were Senger:** Conceptualization, Investigation, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Cedric P. Owens:** Conceptualization, Investigation, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111996.

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