ORIGINAL RESEARCH PAPER

Preparation of high-quality sunflower seed protein with a new chlorogenic acid hydrolase from *Aspergillus niger*

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Abstract

Objective   To investigate the biochemical and enzymatic properties of chlorogenic acid hydrolase (ChlH) from *Aspergillus niger* SD14.721 and its applicability in sunflower seed protein processing.

Results   The ChlH with two identical subunits (97 kDa) was highly stable. Its optimal temperature and pH were determined as 60 °C and pH 7.0. The K_m towards chlorogenic acid (CGA) was 1.85 µM. Based on its *N*-terminal sequence (AVDSVDAIFA), the purified ChlH appeared to be a new chlorogenic acid hydrolase. When applied in sunflower seed protein extraction, ChlH removed 99.13% of CGA in sunflower seed pastes, thus the colour of sunflower seed protein (SSP) changed from green to grey and its visual acceptance improved. Meanwhile, the solubility, water absorption capacity, and emulsification stability of SSP were increased 48.39%, 59.32% and 22.92%, respectively.

Conclusions   A new ChlH was obtained and its feasibility as a CGA-removal tool to obtain high quality SSP was demonstrated.

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Introduction

Chlorogenic acid exists as a soluble ester conjugate of quinic acid and hydroxycinnamic acid, such as ferulic, caffeic and coumaric acids (Butiuk et al. 2015) It can be found in coffee, apples and sunflower seeds at high concentrations (Gonzálezpérez et al. 2002). Chlorogenic acid hydrolase (also named as chlorogenic acid esterase, ChlE) has been attracting commercial attention due to its ability to hydrolyze, esterify and transesterify with 2-phenylethyl alcohol to 2-phenylethyl caffeate (Kakino et al. 2003; Kishimoto et al. 2005). The hydrolysis of chlorogenic acid by ChlH also provided a feasible method to prevent quinone formation, since CGA might covalently combine with the polar groups of proteins under alkaline conditions, which forming green trihydroxy benzacridine derivatives results in significant greening of protein (Liang et al. 2017). In addition, ChlH could enhance the flavour of coffee when roasted by reducing the dehydration and cyclization of CGA to generate chlorogenic acid lactones, which have been identified as key contributors to the bitterness of coffee (Kraehenbuehl et al. 2017; Moon et al. 2009).

Some microorganisms that synthesize ChlH have been reported, including *Arbuscular mycorrhizal* (Negrel et al. 2016), *Aspergillus japonicus* (Okamura and Watanabe 1982), *Aspergillus niger* (Asther et al. 2005; Benoit et al. 2007; Schöbel and Pollmann 1980), *Aspergillus sojae* (Adachi et al. 2008), *Rhizoctoina solani* (Nieter et al. 2017) and *Ustilago maydis* (Nieter et al. 2015, 2016). Moreover, there are also other enzymes that have activities towards CGA, such as FABE (Levasseur et al. 2004), TsFaeC (Vafiadi et al. 2006), Fae1A (Qi et al. 2011), AoFaeB and AoFaeC (Koseki et al. 2009). However, most of these enzymes have various defects, such as the low expression and activities of the wild type strains, the poor stability and weak affinity, which makes the enzymes unacceptable for use in industry.

Our previous research on screening microorganism strains with CGA as the sole carbon source, found that a high-performance strain of *A. niger* SD14 with a CGA hydrolytic activity of 0.048 U/ml, was identified. *A. niger* SD14.721 (0.081 U/ml) was the most optimal producer of ChlH by using atmospheric and ambient-temperature plasma mutagenesis. In this study, we explored the purification and characterization of the extracellular ChlH from *A. niger* SD14.721 and its application on CGA hydrolysis during the sunflower seed protein (SSP) preparation.

Materials and methods

Medium and fermentation conditions of *A.niger* SD14.721

A. niger SD14.721 was obtained from a honeysuckle planting base (Linyi, China) using atmospheric and room-temperature plasma mutagenesis. The GenBank accession number of its gene sequence was KY860764 and it was deposited in *the Culture and Information Centre of Industrial Microorganisms of China Universities* (Wuxi, China.) numbered F0920. The strain was grown in PDA media and fermented to produce enzymes in medium containing (per litre): 5% (w/v) wheat bran as a hydrolase activity inducer, beef extract (2.1 g) and ammonium sulfate (0.93 g) as a compound nitrogen source, maltose (2.5 g) as a carbon source, KH_2PO_4 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.0132 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g. The pH of the liquid medium was adjusted to 6.0, and the strains were fermented at 160 rpm at 25 °C for 11 days.

Biochemical properties of ChlH

Enzyme activity assay

The activity of ChlH was determined by monitoring the CGA decrease at its peak time (8 min) with HPLC (Alliance 2695). The samples (250 μl crude enzyme solution + 100 μl 50 mM phosphate buffer (pH 7) + 650 μl 0.5 mg/ml CGA) were incubated at 60 °C for 15 min and stopped by adding 1 ml methanol. Then the samples were centrifuged, filtered and injected into a Waters Xbrige C_{18} column (4.6·← 250 mm; Waters Corporate, USA) at 30 °C, and eluted with acetonitrile and 2% acetic aqueous solution at a ratio of 15:85(v: v) with a flow rate of 0.5 ml/min. The elution was monitored by a PDA detector at 327 nm. One unit of ChlH activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μM CGA per minute at 60 °C in 50 mM phosphate buffer at pH 7 (Buffer A).

Enzyme purification

After 11 days fermentation, the broth of *A. niger* SD14.721 was filtered and concentrated eightfold by ultrafiltration through a PLCCC membrane (molecular mass cut-off of 5 kDa). The supernatant was then added with ammonium sulfate from 50 to 90% saturation and centrifugated at $5000 \times g$ for 30 min at 4 °C. Then, the precipitate was dissolved in 20 mM Tris-HCl buffer at pH 7.5 (Buffer B) and dialysed against the same buffer. For further purification, the dialysed enzyme was applied to a HiTrap DEAE-Sepharose Fast Flow column (0.7·← 2.5 cm; GE Healthcare, USA), equilibrated with Buffer B and eluted with a 0–0.2 M NaCl linear gradient (pH 7.5) at 1 ml min^{-1} by using the same buffer. The fractions were collected and its activity as well as protein content was assayed. The active fractions were pooled and concentrated to 5 ml. Then the concentrate was applied to a Superdex 200 10/300 GL column (1.0·← 30 cm; GE Healthcare, USA) (Stefan et al. 2018), equilibrated and eluted at a flow rate of 0.4 ml min^{-1} with Buffer B.

Protein analysis

The protein content of enzyme extract was measured according to the Bradford (1976) method with bovine serum albumin as the standard. The purity and the molecular mass were determined by SDS-PAGE electrophoresis (12% resolving gel and 5% stacking gel) (Yang et al. 2018) and size-exclusion chromatography (Superdex 200 10/300GL filtration column). the gel was stained with Coomassie Brilliant Blue R-250. For enzyme protein identification, the first 10 amino acids of the N-terminus were sequenced by Sangon Biotech. Co., Ltd. (Shanghai, China).

Enzyme properties of ChlH

Optimal temperature, pH and stability of ChlH

To explore the optimal temperature, the activity of ChlH was determined at temperature from 20 °C to 80 °C with 50 mM phosphate buffer at pH 7. The thermal stability was evaluated by assaying the residual activity after incubating ChlH at different temperature (20–70 °C) for 30–210 min. Besides, residual activity of the ChlH at 30 °C was assayed at a pH range from 3.0 to 10.0 with a series of buffers, including 50 mM citrate buffer (pH 3.0–5.0), phosphate buffer (pH 5.0–8.0), and glycine–NaOH buffer (pH 9.0–10.0), to determine the optimal pH. The pH stability of ChlH was assayed at the same pH range for 210 min. The enzyme solution without incubation was used as the blank. the reaction was terminated using ice-water bath.

Effect of metal ions on the enzyme activity

The effect of metal ions on ChlH hydrolysis activity was investigated by incubating enzyme solutions at 30 °C for 60 min with 5 mM and 10 mM metal ions, including K^+ (KCl), Na^+ (NaCl), Ca^{2+} (CaCl$_2$), Cu^{2+} (CuSO$_4$), Fe^{2+} (FeSO$_4$), Mn^{2+} (MnSO$_4$), Zn^{2+} (ZnSO$_4$), Mg^{2+} (MgSO$_4$), Al^{3+} (AlCl$_3$) and Fe^{3+} (Fe$_2$(SO$_4$)$_3$) (Song et al. 2018). The enzyme activity without addition of metal ions was used as the control and set as 100%.

Kinetic parameters

The kinetic parameters of ChlH were explored by assaying the initial reaction rates with CGA at 60 °C and pH 7 for 15 min. The K_m and V_{max} values were calculated using double reciprocal Lineweaver–Burk plots. Different concentrations of CGA from 0.001 to 1 mM were used for enzyme reaction rates assay.

Application of ChlH in the preparation of SSP

Phenol-removal treatments and the preparation of SSP

Unshelled intact sunflower seed was purchased from Mengbiyuan Technology Co., Ltd, Inner Mongolia Autonomous region. The sunflower seeds were treated by two different pre-treatments to remove phenol. (a) AS: 100 g seeds were incubated in 1 mM HCl for 3 h (acid soaking) at a solid/liquid ratio of 1:5 (w:v) at 70 °C. (b) CHAS: After acid soaking, these phenol-removed seeds were dried at 60 °C and ground to pastes. 100 g sunflower seed pastes were incubated in 1 l deionized water with 1 ml purified ChlH (0.5 U/ml) added at 60 °C and stirred at 150 rpm for 3 h.

After three different phenol-removal pre-treatments (untreated, AS and CHAS), the aqueous extraction processing (Li et al. 2016) was applied to extract oil from sunflower seed pastes. The oil extraction processing was carried out at 70 °C and pH 9.0 with seed/water ratio of 1:5 (w/v) for 1 h and the oil yield of 87.12 ± 0.41% was obtained. The SSP was prepared from the defatted sunflower seed meals by extraction with alkali (pH 10.5, 60 °C) for 1 h and precipitation at pH 4.5.

Removal yield of CGA

Sunflower seed pastes after different phenol-removal treatments were soxhlet extracted with methanol at 70 °C for 10 h. The CGA in the extracts was quantified using HPLC at 327 nm.

Functional properties

The solubility of SSPs was evaluated as described by Stephanie and Abdullaha (2009) with minor modifications. A portion of SSP samples (0.20 g) was added to 20 ml 0.2 M phosphate buffer (pH 7.0) and

continuously stirred for 30 min. It was subsequently centrifuged at $4000 \times g$ for 15 min.

$$\text{Solubility}\,(\%)$$
$$= \frac{\text{The protein content in supernatants (g)}}{\text{The protein content in protein samples(g)}} \times 100$$

The water absorption capacity (WAC) and fat absorption capacity (FAC) of SSPs were measured as described by Madhusudan and Singh (1985). 8 ml deionized water or soybean oil was added to 0.20 g protein sample (G_1), shaken for 2 min to achieve full adsorption and centrifuged at $6000 \times g$ for 15 min. The rest of the sample was weighed without the supernatant (G_2).

$$\text{WAC/FAC}(\%) = \frac{G_1 - G_2}{\text{The weight of protein samples (g)}}$$
$$\times 100$$

The determination of the emulsification ability index/emulsification stability (EAI/ESI) of SSPs was based on the method of Pearce and Kinsella (1978). Briefly, 100 mg protein sample was dissolved in 25 ml 0.2 M phosphate buffer (pH 7.0) with 10 ml soybean oil added and quickly whipped at 13500 rpm for 1 min. Immediately, 100 μl emulsion of the bottom was pipetted, and 0.1% (w/v) SDS was added, then the absorbance value of the diluted sample was measured at 500 nm (A_0). The emulsified sample was incubated at 30 °C for 60 min and measured (A_{60}) in the same manner as A_0. DF was the dilution factor and C was the protein concentration (g/ml).

$$\text{EAI}\left(\text{m}^2/\text{g}\right) = \frac{6.451 \times A_0 \times DF}{C \times 1000}$$

$$\text{ESI}(\text{min}) = \frac{A_0}{A_0 - A_{60}}$$

Statistical analysis

All experiments were carried out in triplicate, and each result was expressed as the mean ± standard deviations (SD). The differences between groups were tested by one-way ANOVA and Duncan's multiple range tests (SPSS version 19.0) with the level of statistical significance set at $p < 0.05$.

Results and discussions

Enzyme Purification

The enzyme with CGA hydrolysis activity was purified to homogeneity by four separation steps (Table 1) with a high recovery of 29.26%. Ammonium sulfate precipitation could remove most impurity proteins without significant enzyme activity loss. The fractions eluted from the DEAE-Sepharose FF column contained two major protein peaks (Peak 1 and Peak 2), but only Peak 2 had activity against CGA (Fig. 1). This chromatographic strategy did not separate the enzyme effectively due to the broadening of the peaks. All of the fractions that had CGA hydrolysis activity were pooled and applied to a Superdex 200 10/300GL column to further purify the enzyme based on its molecular size. The specific activity of ChlH was 6.15 U/mg with an increase of approximately 547%. The analysis of SDS-PAGE revealed the purified enzyme to be homogeneous (Fig. 2).

Molecular mass and N-terminus

The molecular mass of microbial ChlE monomer was in a narrow range of 70–90 kDa, but with quite different aggregation structure (Table 2). For the Superdex 200 10/300GL gel chromatography, a standard curve was established as a linear relationship between the elution volume (E_V) and the logarithm of the molecular mass (M_W): $\log_{10} M_W = 0.3842 E_{v-} + 2.4227$ ($R^2 = 0.996$). According to this equation, the molecular mass of ChlH in this study was determined as 200 kDa. Relationship between relative mobility (M) and the molecular mass (M_W) in the SDS-PAGE analysis was as following: $\log_{10} M_{W-} = -1.3372 M + 2.1110$ ($R^2 = 0.993$). The SDS-PAGE analysis of enzymes showed a single band after gel separation, and the subunit molecular mass was calculated as 97 kDa (Fig. 2). Therefore, ChlH was a dimer with two identical subunits, which was slightly bigger than that of the monomeric UmChlE of *U. maydis* (71 kDa) (Nieter et al. 2015) and the tetrameric ChloE of *A. niger* CBS 513.88 (4 × 60 kDa) (Schöbel and Pollmann 1980). Both its weight and aggregation structure were more similar to the chlorogenic acid hydrolases of *A. sojae* (2 × 88 kDa) (Adachi et al. 2008) and *A. niger* BRFM 131 (2 × 80 kDa) (Asther et al. 2005).

Table 1 Purification of chlorogenic acid hydrolase from fermented broth of *Aspergillus niger* SD14.721

Purification step	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
Supernatant of fermented broth	1600	40.99	39.09	0.95	100	1
Ultrafiltration	200	36.77	37.22	1.01	95.22	1.06
$(NH_4)_2SO_4$ precipitation	99	6.24	17.99	2.88	46.02	3.03
DEAE-Sepharose FF	160	3.64	13.04	3.58	33.35	3.76
Superdex 200 10/300GL	25	1.86	11.44	6.15	29.26	6.47

The enzyme in the supernatant of fermented broth was used as the control and set as 100%
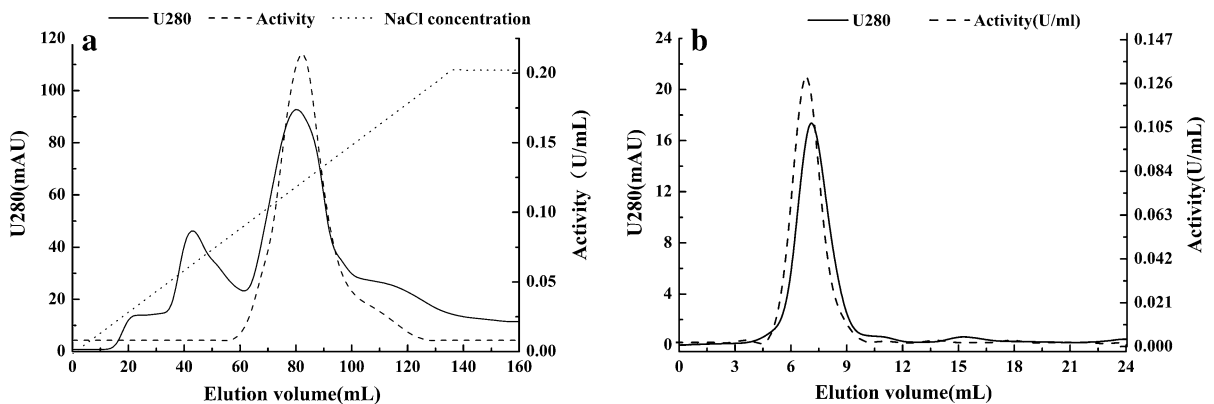

Fig. 1 Elution curve of enzyme samples on different chromatography. **a** DEAE-Sepharose F. F anion exchange chromatography; **b** superdex 200 10/300GL size exclusion chromatography

Enzyme properties

The enzyme properties of the purified ChlH were shown in Fig. 3. The optimal temperature of ChlH was 60 °C (Fig. 3a), and the optimal pH was determined as 7.0 at 60 °C (Fig. 3c). The enzyme remained stable between pH 3.0–7.0 at 20 °C for 210 min (Fig. 3b). The enzyme was also resistant to change at 20–50 °C (Fig. 3d). Compared to reported ChlEs (Table 2), ChlH obtained in this study also showed relatively better stability. During the subsequent processes of oil extraction (at pH 9.0 and 70 °C for 3 h), the ChlH was inactivated due to pH change.

Among all tested metal ions (K^+, Na^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+}), none promoted its hydrolytic activity towards CGA. Monovalent metal ions decreased the activity slightly. However, except for Mg^{2+} and Ca^{2+}, divalent and trivalent metal ions remarkably inhibited the activity of ChlH. These different inhibition effects on enzyme
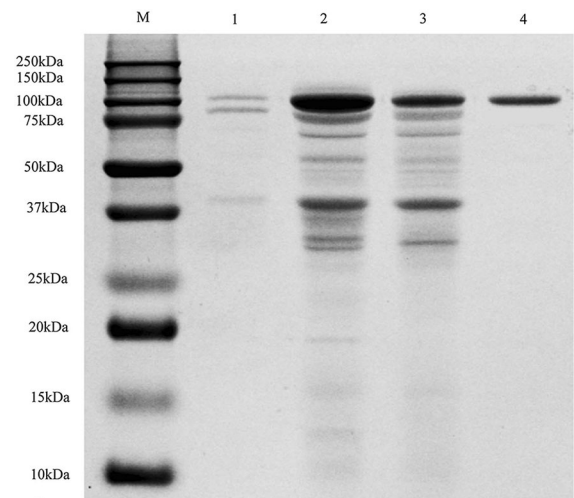

Fig. 2 SDS-PAGE analysis of the ChlH during its purification. Lane M: protein molecular size marker; Lane 1: the enzyme in *A.niger* SD17.421 culture medium; Lane 2: the enzyme solution after ultrafiltration; Lane 3: the enzyme solution after $(NH_4)_2$-SO_4 fractionation; Lane 4: the enzyme after DEAE-Sepharose F.F and Superdex 200 10/300GL chromatography

Table 2 The comparisons between the ChlH and other enzymes with hydrolysis activity towards chlorogenic acid

Name	Specific activity (mU/mg)	Recovery (%)	Aggre-gation states	Molecular mass (kDa)	pH optima	pH stability	Optimal temperature (°C)	Thermal stability (°C)	K_m (μM)	V_{max} (μM/mg·s)	Source and reference
ChlH	6.15	29.26	Dimer	97	7	3.0–7.0	60	20–50	1.85	0.38	*Aspergillus niger;* This study
ChloE	8.25	–	Tetramer	60	6.5	3.0–8.5	45	25–55	700	–	*Aspergillus niger;* Schöbel (1980)
ChlE	103.33	2	Dimer	80	6	5.5–7.0	55	20–40	10	0.14	*Aspergillus niger;* Asther (2005)
Recombine ChlE	112	55	Tetramer	74.553	6	3.80–7.85	60	37–50	6.5	0.25	*Aspergillus niger;* Benoit (2007)
Chase	24.22	26	Dimer	88					–	–	*Aspergillus sojae;* Adachi (2008)
UmChlE	237.6	0.3	Monomer	71	7.5	4–10.0	37	–	19.6	–	*Ustilago maydis;* Nieter (2015)

activity could be explained that different metal ions showed different binding abilities to active sites in ChlH, therefore influencing the enzyme structure in different ways. The inhibitions of chlorogenic acid hydrolysis activity by Zn^{2+}, Fe^{2+} and Al^{3+} were consistent with the feruloyl esterase from *Russula virescens* (Wang et al. 2014). The negative effects of Cu^{2+} (Donaghy and Mckay 1997) and Fe^{3+} (Yao et al. 2013) on esterase activity were also described previously.

The purified ChlH had a high affinity for its substrate, and obeyed Michaelis–Menten kinetics over the substrate concentration ranging from 0.001 mM to 10 mM. The K_m and V_{max} values were calculated to be 1.85 μM and 0.38 μM/(mg s) with nonlinear regression using the Lineweaver–Burk plot. The k_{cat} value and the specificity constant (k_{cat}/K_m) were 0.49/s and $2.65 \cdot \leftarrow 10^5$/(mol s), respectively. Compared to reported data (Table 2), ChlH had a stronger affinity towards CGA.

The first 10 amino acids of the *N*-terminus were sequenced at Sangon Biotech. Co., Ltd. (Shanghai, China) and determined to be AVDSVDAIFA. The sequence was submitted to the NCBI database to BLAST (Altschul et al. 1997) with other previously published proteins, and no homologous match was identified. Considering molecular mass, enzymatic properties and kinetic comparisons, the purified enzyme was appeared to be a new chlorogenic acid hydrolase that had not been reported yet.

Application of ChlH in the preparation of SSP

The CGA levels in the sunflower seed pastes after acid soaking and further ChlH treatment were shown in Table 3. After ChlH hydrolysis treatment, the CGA content in sunflower seed were greatly lowered by 99.13%, achieving a nearly complete removal. Previous studies showed that ChlE could hydrolyse other natural substrates, such as coffee pulp and apple marc. The recombinant CholE (0.6 U/g) from *A. niger* could release all the CGA from coffee pulp after overnight incubation (Benoit et al. 2007), and UmChlE (0.02 U/g) from *U. maydis* also liberated 68% of the CGA from coffee pulp after an incubation period of more than 20 h (Nieter et al. 2015). Therefore, for sunflow seed paste which containing high amount of oil, the successful attempt of ChlH would widely broaden its potential application value.
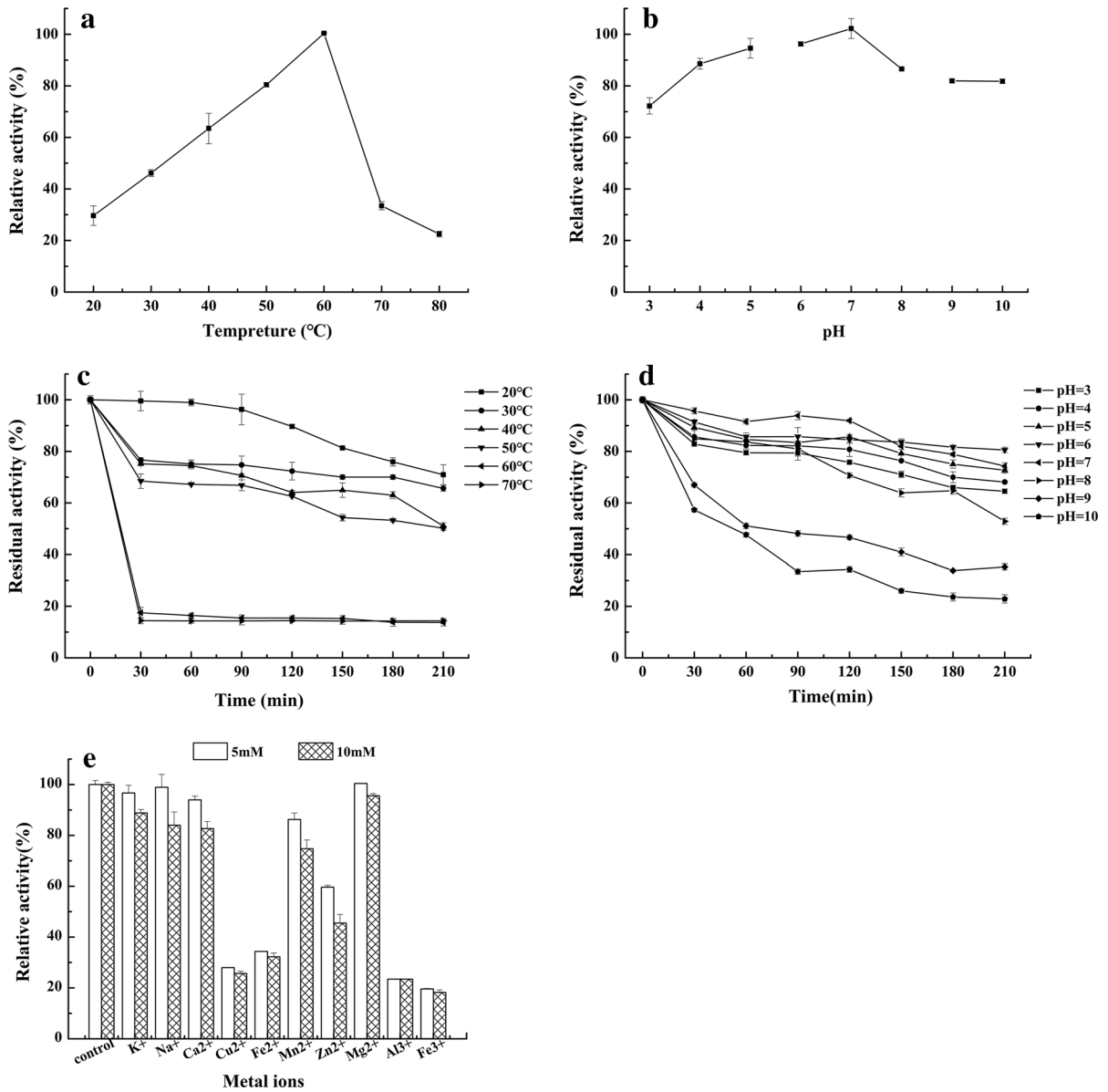
Fig. 3 Enzyme properties of the ChlH. **a** Temperature optima; **b** thermal stability; **c** pH optima; **d** the pH stability, pH in the range of 3.0 to 10.0 by using 50 mM citrate buffer (pH 3.0 to 5.0), phosphate buffer (pH 5.0 to 8.0) and glycine–NaOH buffer (pH 9.0 to 10.0). **e** Effect of metal ions on the activity of ChlH. Values are the averages of data from triplicate experiments, with standard deviations shown as error bars

Table 3 The chlorogenic acid esterase (CGA) content in sunflower seed during phenol-removal processing

Substrate	Treatment	CGA (mg·g^{-1})
Sunflower seed pastes	Untreated	13.82 ± 0.05[a]
	Acid soaking	6.99 ± 0.19[b]
	ChlH hydrolysis after acid soak	0.12 ± 0.03[c]

The data are expressed as the mean $\pm$ SD (n = 3). CGA Means followed by the different letters are significantly different (p < 0.05)

The decolouration effects in the prepared SSP after different phenol-removal treatments were shown in Fig. 4. The functional properties of the three SSPs and soy protein isolate, especially the solubility, WAC/FAC and EAI/ESI were compared and analysed (Table 4). The acid soaking progress decrease the concentration of CGA, thereby the concentration of the CGA-protein, and ChlH hydrolysed a portion of the CGA-protein conjugates to increase the solubility of the SSP.
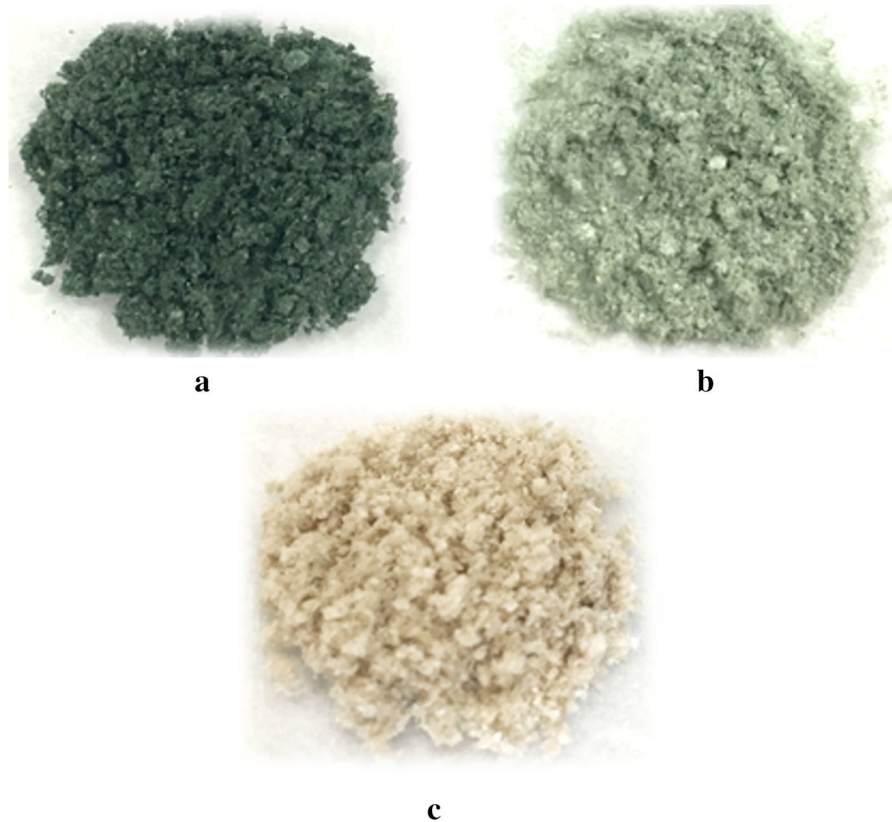

Fig. 4 The color of sunflower seed proteins (SSPs) under different treatment conditions. **a** untreated; **b** acid soaking; **c** ChlH hydrolysis after acid soaking

Table 4 Comparison of functional properties of sunflower seed protein (SSP) and soy protein isolate

Functional properties	Sunflower seed protein			Soy protein isolate
	Untreated	Acid soaking	AHAS	
Solubility (%)	59.1 ± 1.2^c	68.8 ± 0.9^b	87.7 ± 1.1^a	46.1 ± 1.4^d
Water absorption capacity (%)	460.6 ± 13.8^d	582.0 ± 8.7^c	733.82 ± 10.7^b	1080.1 ± 22.3^a
Fat absorption capacity (%)	504.01 ± 18.5^c	518.91 ± 14.8^b	540.5 ± 30.86^a	193.1 ± 4.0^d
Emulsification ability index ($m^2 \cdot g^{-1}$)	93.8 ± 2.4^a	93.7 ± 7.1^a	90.1 ± 6.4^a	79.4 ± 4.0^b
Emulsification stability index (min)	62.4 ± 1.8^c	61.9 ± 0.5^c	76.7 ± 2.2^a	68.9 ± 2.5^b

The data are expressed as the mean $\pm$ SD (n = 3). Means in a row followed by the different letters are significantly different ($p < 0.05$), the same letters mean show no significant difference ($p > 0.05$)

AHAS ChlH hydrolysis after acid soak

For the first time, the feasibility of using chlorogenic acid hydrolase as a pre-processing method to remove CGA from sunflower seed was demonstrated. The newly industrial application of aqueous extraction processing on oilseed like peanut shows very promising future as a new way for comprehensive usage of both its oil and protein (Li et al. 2016). By applying the ChlH in sunflower seed processing, high-quality SSPs could be recovered using aqueous extraction processing.

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