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Dual-upgraded biomass saccharification and bioethanol production by cascading peptides interactions with polymers and activating cellulases in bioenergy crops

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ABSTRACT

Crop lignocelluloses represent enormous biomass resources convertible for biofuels and bioproducts, but efficient biomass saccharification is crucially required for large-scale biofuel production. Although plant proteins and chemical surfactants could enhance biomass saccharification, it remains to explore advanced activator for further improving lignocellulose degradation and conversion to biofuels. In this study, soybean protein and its peptides were employed into the mixed-cellulases hydrolyses of distinct lignocellulose substrates from representative bioenergy crops. Significantly, the optimal peptides supply could continuously upgrade the hexoses and bioethanol yields by 20 % and 35 %. Despite the soybean peptides, like plant proteins and Tween-80, could effectively interact with lignin and xylan for unblocking mixed-cellulases incubated, this study found a unique interaction between peptides and cellulose microfibrils to retain the mixed-cellulases more accessible for facilitating lignocellulose hydrolysis, which caused either significantly higher hexoses yield or more improved sugar conversion to bioethanol, compared to soybean protein and Tween-80. Based on all findings achieved, this study finally proposes a novel mechanism model about how green peptides act as both active biosurfactant and enzyme activator for cascading improvements of lignocellulose enzymatic degradation and yeast fermentation, thereby providing a powerful strategy applicable for green-like biomass saccharification and highly-efficient bioethanol production in bioenergy crops.

1. Introduction

Plant cell walls represent abundant polymeric biomasses convertible for advanced biofuels and valuable bioproducts, thereby aiding to reduce net carbon release and globe warming [1,2]. However, the native recalcitrance of lignocellulose crucially obstructs biomass enzymatic saccharification, leading to a costly conversion to biofuels together with unavoidable secondary wastes release into the environment [3,4].

Lignocellulose recalcitrance is profoundly directed by plant cell wall composition, wall polymer aspect and wall-network pattern [5]. To reduce the recalcitrance, physical and chemical pretreatments have been executed to partially extricate non-cellulosic polymers (lignin, hemicellulose) and to specifically lessen cellulose crystallinity and polymerization [6,7]. Particularly, the alkali pretreatment could effectively co-extract hemicellulose and lignin in the most bioenergy crops examined [8,9]. As cellulosic ethanol has been deemed as an outstanding additive into the petrol fuels, biomass enzymatic saccharification is considered as the crucial step to upgrade bioethanol productivity by employing active lignocellulose-degradation enzymes including exoglucanases (CBHs), endoglucanase (EGs), β-glucosidases

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(BGs) and xylanases [10,11]. Because such mixed-cellulases could be simply adsorbed with lignin and hemicellulose, high dosages of enzymes cocktails are in principle required to load for biomass enzymatic saccharification [12,13]. Meanwhile, large amounts of toxic compounds could be generated from both biomass pretreatment and enzymatic saccharification, which consequently inhibit final yeast fermentation with relatively low sugar-ethanol conversion rate [14]. Therefore, it becomes important to explore green-like biotechnology applicable for further enhancing biomass saccharification and bioethanol production.

The mixed-cellulases are the crucial biocatalysts that convert carbohydrates in lignocellulosic biomass into fermentable sugars [15]. Although genetic engineering of fungi strains could improve the secretion of mixed-cellulases, it is hard to find out the optimal proportion among four types of enzymes, due to diverse and complicated lignocellulose structures and functions of major bioenergy crops [16]. Alternatively, surfactant-like additives are increasingly implemented for widespread enhancements of biomass enzymatic saccharification [17]. Particularly, plant proteins and chemical surfactants are co-supplied to improve cellulose enzymatic hydrolysis by effectively blocking cellulases adsorption with lignin and hemicellulose [18]. For example, *Amaranthus* green proteins and Tween-80 have been respectively examined with highly adsorptive capacities with lignin to remarkably enhance lignocellulose enzymatic hydrolysis for high-yield bioethanol production in bioenergy crops examined [19,20].

Soybean plant stands out as a rich protein source for animal feed after oil extraction [21]. As soybean protein is of amphoteric property for active interaction with bioactive molecules, it has been characterized as effective biosurfactant for enhancing lignocellulose enzymatic digestion [22,23]. Meanwhile, soybean protein is digested to produce the peptides as potential nutritional ingredients in food, medicine, cosmetics and others [24]. However, little has been yet investigated about protein peptides roles in biomass enzymatic saccharification and bioethanol production.

By employing distinct lignocellulose substrates obtained from alkali pretreatments with three representative bioenergy crops (*Miscanthus*, barely, poplar), this study initially co-supplied different dosages of soybean protein and peptides with mixed-cellulases to test the enhancement roles in biomass enzymatic saccharification. By comparison with chemical surfactant (Tween-80) and soybean protein, the soybean peptides were identified as the optimal biosurfactant for dual enhancements of biomass saccharification and bioethanol production. Accordingly, this work detected the dual interactions of soybean peptides with lignin and cellulose, being different from soybean protein and Tween-80, and thus explored a novel function about the simultaneous lignin interaction and cellulases co-activation with the peptides. Finally, this study proposed a hypothetic model to address the findings about soybean peptides as super alternatives to soybean proteins and Tween-80 for the hexose and ethanol yields increased by 20 % and 35 % via dual mechanisms: (1) Soybean peptides universally prevent the cellulases from lignin adsorption; (2) Soybean peptides uniquely interact with cellulose microfibrils to facilitate cellulases hydrolytic activity.

2. Materials and methods

2.1. Biomass, enzymes and microorganisms

Three types of lignocellulose samples (*Miscanthus*, barley, poplar) were respectively collected from Huazhong Agricultural University experimental station. The biomass samples were dried at 50 °C, ground into powders through a 40 mesh sieve and stored in a dry container until use. The mixed-cellulases enzymes (HSB) was obtained from Imperial Jade Biotechnology Co., Ltd. Ningxia, China. The commercial enzymatic cocktail contained β-glucanase ($\geq 3.60 \times 10^4$ U), cellulase ($\geq 3.60 \times 10^2$ U) and xylanase ($\geq 6.00 \times 10^4$ U). Soybean protein and pepsin were purchased from Xiya Chemical Technology (Shandong) Co., Ltd, and Shanghai Macklin Biochemical Co., Ltd., respectively.

2.2. Wall polymer extraction and determination

Wall polymer fractionation was accomplished as previously described [25,26]. Cellulose content was estimated by determining hexose of the cellulose fraction using the anthrone/H_2SO_4 method. Total hemicelluloses were calculated by determining all hexoses and pentoses using the orcinol/HCl method. Lignin content was measured by the two-step acid hydrolysis method according to the NREL's laboratory analytical protocol [27]. All experimental analyses were performed in biological triplicate.

2.3. Biomass chemical pretreatments

H_2SO_4 pretreatment: The well-mixed biomass samples of *Miscanthus*, barley and poplar were respectively treated with 6 mL H_2SO_4 at different concentrations (1 %, 2 %, 3 %, 4 %, 6 %, 8 % v/v). The samples were sealed and heated at 121 °C for 20 min in autoclave (0.15 Mpa). After centrifugation at 3000*g* for 5 min, all supernatants were collected for pentoses and hexoses assay [28,29].

NaOH pretreatment: The well-mixed biomass samples were incubated with 6 mL NaOH at various concentrations (0.5 %, 1 %, 1.5 %, 2 %, 3 %, 4 %, 6 %, 8 %, w/v) under 150 rpm shaken at 50 °C for 2 h. The remaining pellets were washed with 10 mL distilled water for 5–6 times until pH 7.0. Samples were only added with 6 mL distilled water and shaken for 2 h at 50 °C as control [30].

2.4. Preparation of soybean peptides

Pepsin solution was prepared freshly for each assay by dissolving pepsin with HCl solution (pH = 3.0) as described below. About 200 μL HCl solution was added into 2 mL plastic tubes consisting of 0.036 g pepsin, and 100 μL pepsin solution was incubated with 0.36 g soybean proteins at 50 °C with continuous shaking for 3 h. The ratio of pepsin to soybean protein was at 1:20 on a weight basis [31,32]. After boiling water for 10 min to stop the reaction, soybean peptides were neutralized and collected until in use. About 100 μL inactivated pepsin was added into 0.36 g soybean proteins as the control. The experiments were conducted under independent biological triplicate.

2.5. Yeast fermentation

Saccharomyces cerevisiae strain (Angel yeast Co., ltd., China) was used for fermentation testing [33]. Angel yeast cells were cultured for 2 days in 250 mL YPD (80 g/L, 160 g/L and 320 g/L glucose) with 12 % soybean protein/peptides at 37 °C with orbital shaking at 150 rpm, cell density was analyzed from turbidity readings at 600 nm after dilution with water.

2.6. Biomass enzymatic saccharification and ethanol measurement

Biomass enzymatic saccharification and yeast fermentation were respectively conducted as previously described [34]. The pretreated lignocelluloses were incubated with 0.20 % (w/v) mixed-cellulases (HSB) with final concentrations of cellulases at 10.60 FPU/g biomass and xylanase at 6.72 U/g biomass with 5 % solid loading [35]. The enzymatic hydrolysis was performed at 50 °C under 150 rpm shaken. The released hexose and pentose were measured by anthrone/H_2SO_4 and orcinol/HCl methods, respectively. *S. cerevisiae* strain was inoculated with the enzymatic hydrolysates [36]. The fermentation solution was distilled for the determination of ethanol content. The ethanol content was measured by the $K_2Cr_2O_7$ Method [37]. All assays were accomplished at independent triplicate.

2.7. Cellulase activity

Filter paper activity/FPA was determined for cellulase activity assay

by incubating 2 g/L commercial mixed-cellulases (HSB) with 50 mg Whatman filter paper co-supplied with 12 % soybean protein or 12 % soybean peptides at phosphate buffer (0.05 M, pH 4.8) for 48 h at 50 °C as previously described [38]. The well-mixed sample was incubated for 60 min at 50 °C, and the reaction was stopped by adding 2 mL DNS followed by boiling water for 10 min. One FPA unit was defined by measuring the amount of enzyme releasing 1 μmol reducing sugar per min from Whatman filter paper grade No.1. All proteins in supernatants were detected by Bradford assay using UV–vis spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai). All assays were accomplished at independent triplicate.

2.8. Protein binding assay

Protein binding assay was conducted as previously described [39]. About 5 mg soybean protein/peptides (per mL) was incubated in 0.2 M Na-acetate buffer (total volume of 2 mL, pH 4.8) with 50 mg commercial lignin, xylan and Avicel/cellulose. After shaken at 25 °C for 12 h, the samples were centrifuged at 4000×*g* for 5 min. All proteins in supernatants were detected by Eppendorf BioPhotometer. All assays were accomplished at independent triplicate.

2.9. SDS-PAGE analysis

SDS-PAGE was operated using Stain-Free precast gels (Beijing Zoman Biotechnology Co., Ltd.) according to the manufacturer's instruction. About 30 μL supernatant were respectively loaded into SDS gels. Protein samples were visualized with colloidal Coomassie blue staining as previously described [40].

2.10. Zeta potential and surface tension measurement

Soybean protein and peptides were respectively incubated with biomass substrates of *Miscanthus*, barley and poplar samples for 12 h adsorption. The zeta potential of the supernatants was detected by a Dynamic Light Scattering (DLS) Analyzer equipped with a laser Doppler microelectrophoresis (Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK) as previously described [41]. The samples were filtered by 0.45 μm syringe membranes (Millipore, Billerica, MA, USA) for analysis and deionized water was used for background correction. All zeta potential measurements were performed under independent triplicate.

The surface tension γ of various solutions were performed via a NIMA ST-9000 tensiometer (Nima Technology, Coventry, UK) as previously described [18,42]. Each sample was measured until γ became constant, which was after at least 10 min to ensure the saturation of surfactant adsorption on the air/water interface. The calibration reference was the γ of Milli-Q water was measured to ensure accuracy. The surface tension of the soybean protein and soybean peptides solution were measured immediately and maintained at an ambient temperature of approximately 23 °C.

2.11. Statistical analysis of correlation coefficients

Statistical analysis was performed using IBM SPSS Statistics software with analysis of variance (ANOVA), and the data were presented as mean ± SD with significant difference at the 95 % confidence level ($p < 0.05$). Student's *t*-test was performed using the IBM SPSS Statistics software. All experimental assays were accomplished at independent triplicate unless indicated.

3. Results and discussion

3.1. Soybean peptides are constantly effective for enhancing biomass enzymatic saccharification in bioenergy crops

Given plant proteins play a surfactant-like role for lignocellulose enzymatic hydrolyses [18], this study firstly attempted to explore if protein peptides are also effective for enhancing biomass enzymatic saccharification (Fig. 1). By employing the peptides of soybean protein obtained from classic pepsin digestion, we respectively supplied different dosages of soybean proteins and its peptides into the enzymatic hydrolyses of NaOH-pretreated lignocellulose substrates in three representative agriculture and forestry plant species such as *Miscanthus*, barley and poplar (Fig. 1A). Even though those three bioenergy crops contain distinct lignocelluloses in their raw materials and alkali-pretreated residues (Table 1), this study determined consecutively raised hexoses yields (% cellulose) released from mixed-cellulases hydrolyses, while either soybean protein or peptides are increasingly co-suppled (Fig. 1B–D). Particularly, the peptides supply could cause consistently higher hexoses yields than the soybean protein did, and much more hexoses yields were obtained from the optimal dosage (12 %) supplement in three bioenergy crops examined. Meanwhile, we performed commonly acid and alkali pretreatments using different concentrations of H_2SO_4 and NaOH to determine hexoses yields released from mixed-cellulases hydrolyses without soybean protein and peptides supply (Fig. S1). Despite the acid and alkali pretreatments at high concentrations could enhance biomass enzymatic saccharification in three bioenergy crops, their hexoses yields were still lower than those of the dilute-alkali pretreated lignocelluloses co-supplied with the optimal peptides, indicating that the peptides supply should be a green-like technology for much more enhancement of biomass saccharification via less acid/alkali application for biomass pretreatment. Since the peptides solution contains the pepsin applied for soybean protein digestion, we checked that the co-supplement of inactivated pepsin had little impact on lignocelluloses hydrolyses, but active pepsin loading could completely stop biomass enzymatic saccharification, which should be due to the mixed-cellulases digestion by pepsin (Table S1). As soybean protein digestion could produce small amounts of amino acids, this study also co-supplied four major kinds of amino acids into the mixed-cellulases reaction, leading to slightly reduced hexoses yields from enzymatic hydrolyses of alkali-pretreated lignocelluloses (Table S2). Because Tween-80 has been well defined as active chemical surfactant applicable for enhancing biomass enzymatic saccharification [19,20], this study also co-supplied the optimal Tween-80 into the enzymatic hydrolyses of alkali-pretreated lignocelluloses, and then determined slightly higher hexoses yields than those of the peptides-supplied samples in three bioenergy crops (Fig. 1E–G). Therefore, the results revealed that the soybean peptides should play a specific enhancement role in biomass enzymatic saccharification.

3.2. Soybean peptides are optimal for maximizing bioethanol production

As the optimal supplements of soybean protein/peptides and Tween-80 could respectively enhance biomass enzymatic saccharification under NaOH pretreatments as described above, this study consequently performed yeast fermentation for bioethanol production in two bioenergy crops (Fig. 2). Compared to the control (without proteins/peptides/Tween-80 supplement), only peptides supplement could significantly increase bioethanol yield by 23 % at $p < 0.05$ level (n = 3) in *Miscanthus* samples, whereas the supplements of soybean protein, peptides and Tween-80 led to significantly raised bioethanol production by 24 %, 35 % and 27 % in barley samples, respectively (Fig. 2A and B). Consistently, the peptides supply remained to achieve higher bioethanol yields than the soybean protein and Tween-80 did in all *Miscanthus* and barley samples examined. Furthermore, the peptides supply could cause the hexose-ethanol conversion rates at 74 % and 75 % in *Miscanthus* and barley samples, whereas either the protein and Tween-80 supply or the control sample showed their hexoses-ethanol conversion rates ranged from 62 % to 72 % (Fig. 2C and D). To understand how the peptide supply improves the conversion rate, this study performed a standard yeast fermentation to observe yeast cell growth by employing three dosages of standard glucose (80, 160, 320 g/L) as sole carbon source

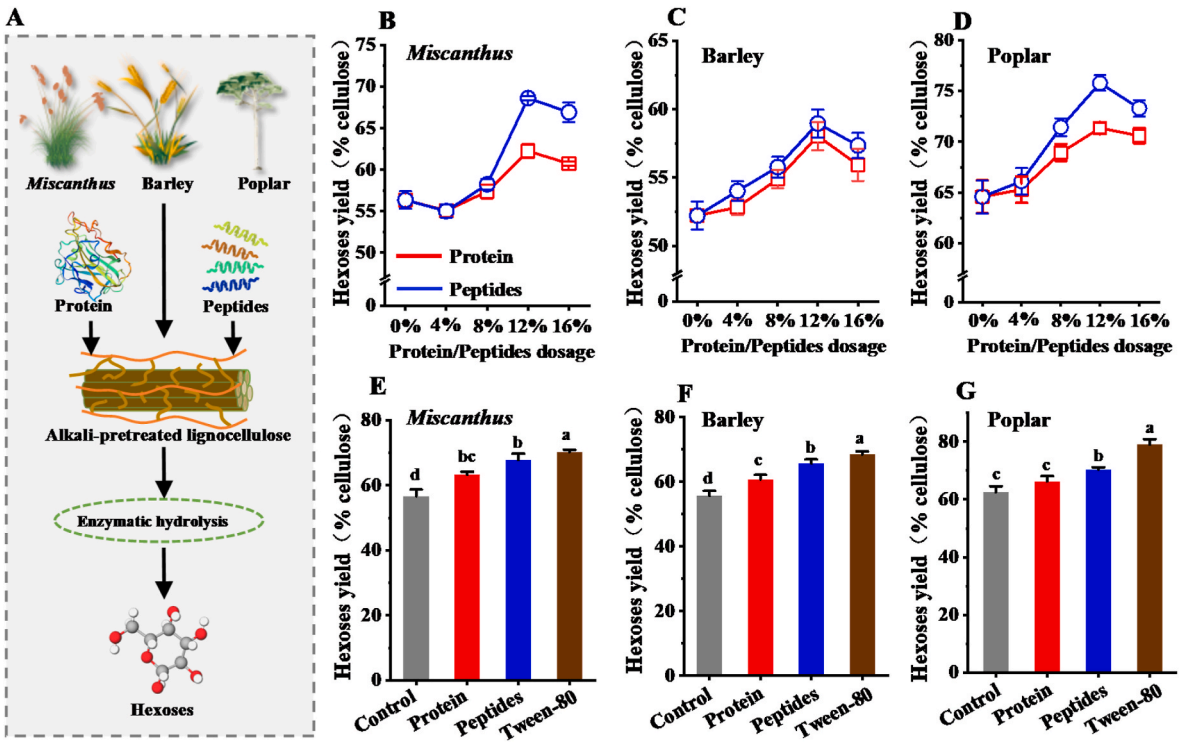

Fig. 1. Comparison of soybean protein and its peptides enhancements for biomass enzymatic saccharification of three representative bioenergy crops. (A) Experimental flow chart; (B, C, D) Hexoses yields released from enzymatic hydrolyses of alkali-pretreated lignocelluloses co-supplied with different dosages of protein and peptides in *Miscanthus* (2 % NaOH pretreatment), barley (1 % NaOH) and Poplar (4 % NaOH), respectively; (E, F, G) Hexoses yields from enzymatic hydrolyses of three lignocellulose samples as shown (B, C, D) co-supplied with 12 % protein or 12 % peptides or 1 % Tween-80; Control without any protein/peptides/Tween-80 supplement; Data as means ± SD (n = 3); The letters (a, b, c, d) highlighted as significant differences among the samples examined by Tukey's test ($p < 0.05$).

Table 1
Lignocellulose compositions of raw materials and NaOH-pretreated residues of three bioenergy crops.

	Cellulose			Hemicellulose			Lignin		
	Raw	NaOH-pretreated		Raw	NaOH-pretreated		Raw	NaOH-pretreated	
***Miscanthus* 2 % NaOH**	36.81 ± 0.44	28.79 ± 1.13	**−21.8 %**[a]	22.29 ± 0.55	15.21 ± 0.22	**−31.80 %**	28.81 ± 0.60	13.28 ± 0.53	**−53.90 %**
Barley 1 % NaOH	43.07 ± 1.12	31.39 ± 0.51	**−27.10 %**	26.51 ± 0.71	14.70 ± 0.12	**−44.60 %**	21.94 ± 0.69	11.14 ± 0.31	**−49.20 %**
Poplar 4 % NaOH	30.92 ± 1.57	30.77 ± 0.87	**−0.50 %**	12.01 ± 0.31	13.30 ± 0.94	**10.70 %**	23.12 ± 0.71	16.28 ± 0.28	**−29.60 %**

Data as means ± SD (n = 3)
[a] As reduced percentage of pretreated lignocellulose relative to the raw material.

(Fig. S2). During a time course incubation, the peptides supply could not significantly alter the numbers of yeast cells compared to the control, revealing that the peptides supply should not impact yeast cell growth. On the other hands, the peptides supply may reduce toxic chemical inhibition to yeast fermentation and/or improve hexose-ethanol metabolism in yeast cells. Despite of relatively higher hexose yield achieved, the Tween-80 supply caused relatively lower hexose-ethanol conversion rates than the peptides supply did, which was validated by mass balance analysis (Fig. S3). This finding was also consistent with the previous reports about relatively more toxic chemical release from Tween-80 supply including acetic acid, furfural, and 5-hydroxymethylfurfural, which can inhibit the growth and development of yeast cells [14,23].

Furthermore, this study compared with other seven biosurfactants supplements from previously reports such as cationic kraft lignin [43], soybean protein [22,44–46], sophorolipid [47], peptone [48], soy skim [49], bovine serum albumin [50] and Tween-80 [51], and the soybean peptide supply performed in this work could even cause relatively more increases of both hexoses and bioethanol yields (Table 2), indicating that the soybean peptides should be one of the optimal biosurfactants for maximizing biomass enzymatic saccharification and bioethanol conversion. Further based on the globe biomass yields harvested from *Miscanthus,* barley and poplar growths, this study estimated total bioethanol production potential from soybean protein and peptide supply (Table 3) [52–54]. By supplying soybean peptides into biomass processes, the *Miscanthus*, barley and poplar could respectively produce the bioethanol at 2.28, 0.64, 0.47 ton per hectare/per year, which were consistently higher than those of the soybean protein supply and the control, providing an applicable strategy to maximize bioethanol production from soybean peptides supply and global *Miscanthus* growth. Despite the soybean peptides supplement enabled more bioethanol production, its processing cost from pepsin digestion should be relatively higher than those of the soybean protein and Tween-80 supplies accounting for an additional $0.0018 per application, which may finally cause less profit according to the minimum sale price of bioethanol at 0.47 $/kg [55]. Thus, it remains to explore recycling of the soybean peptides from bioethanol refinery for value-added bioproducts in the future.

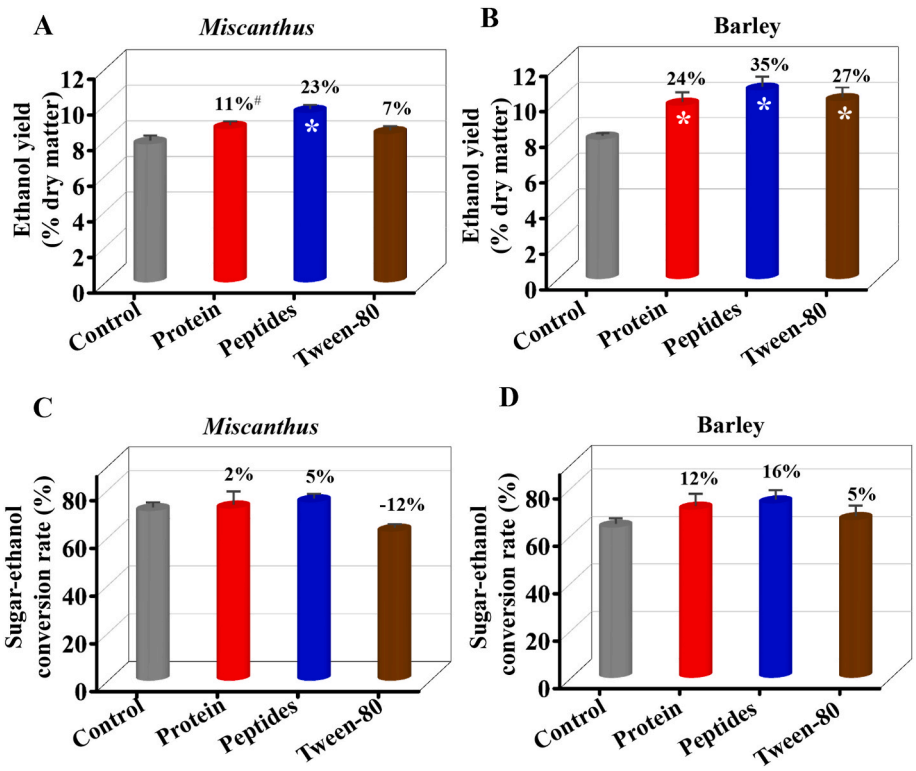

Fig. 2. Comparison of bioethanol productivity by yeast fermentation with hexoses released from enzymatic hydrolyses co-supplied with soybean protein/peptides and Tween-80 in two bioenergy crops. Data as means ± SD (n = 3); * As a significant different ethanol yield or conversion rate relative to the control by *t*-test at $p < 0.05$; # As increased rate of ethanol yield/rate relative to the control.

Table 2
Comparison of hexoses and bioethanol yields achieved from peptides supply in this study and other additives in previous works.

Additives	Biomass (w/v)	Pretreatment	Enzyme loading	Hexose yield increasing Rate (%)	Ethanol yield increasing Rate (%)	Ref.
Peptides	**5 % Barley**	**1 % NaOH**	**(HSB) 10.6 FPU/g**	**18.0**[a]	**35.0**[b]	**This study**
Cationic kraft lignin	10 % Corn	2 % NaOH	Azure Biological (10.0 FPU/g)	11.6	–	[43]
In-house extracted soy protein	15 % Sugarcane bagasse	Liquid hot water	Cellic CTec3 (5.0 FPU/g)	76.0	–	[44]
Soybean protein	20 % Apple pomace	1 % H_2SO_4	Cellic CTec2 (60.0 FPU/g)	24.8	20.9	[45]
Soybean protein	15 % Sugarcane bagasse	Liquid hot water	Cellic CTec2 (5.0 FPU/g)	61.0	86.0	[46]
Soybean protein	5 % Sugarcane bagasse	Steam-exploded	Filamentous fungus Enzyme cocktail	200.0	–	[22]
Sophorolipid	20% Sugarcane bagasse	2 % NaOH	Cellulase, hemicellulase And cellobiase (10.0 FPU/g, 150 U/g, 60 mg/g)	17.8	–	[47]
Peptone	Rice	1M NaOH	*Acremonium cellulase*	13.7	–	[48]
Soy skim	40 % Corn	Cold deionized (DI) water, 80 °C	α-Amylase, glucoamylase	–	20.0	[49]
Bovine serum albumin	2 % Rice	2 % NaOH	*Acremonium Cellulase* (15.0 FPU/g)	–	13.6	[50]
Tween-80	2 % Wheat	1.2 % H_2SO_4	Cellulase and cellobiase (25.0 FPU/g, 2.5 CBU/g)	13.9	–	[51]

[a] As increased rates of hexoses yield (% cellulose) and.
[b] ethanol yield (% dry matter) compared to the control (without additives); - As unavailable data.

3.3. *Soybean peptides are consistent to retain high cellulases activities*

To test the soybean peptides enhancement on biomass enzymatic saccharification, this study conducted a standard assay of cellulases activities *in vitro* (Fig. 3). By incubation with Avicel/cellulose substrate with mixed-cellulases, the supplement of 12 % soybean protein or 12 % soybean peptides could cause consistently higher filter paper activities (FPAs) than those of the control (without soybean protein/peptides) during a time course of reactions from 12 h to 48 h, but the peptides supply remained a higher activity than the soybean protein did (Fig. 3A). Meanwhile, the SDS gel running was performed to separate the major enzymes of mixed-cellulases, and both soybean protein and peptides samples exhibited relatively higher quantities of two major enzymes bands than those of the control after 36 h and 48 h reactions (Fig. 3B; Fig. S4). Based on the semi-quantitation of two major bands accounting for CBHI and EG enzymes [40], the control sample showed much reduced CBHI and EG levels after 48 h incubation, indicating a drastic enzyme self-degradation (Fig. 3C and D). Even though both soybean

Table 3
Estimation of potential global bioethanol yields (t/ha/year) in three bioenergy crops from soybean protein and peptides supply.

Sample	BiomassYield t/ha/year	Bioethanol yield (t/ha/year)								Ref.
		Control	Value ($)	With soybean protein	Value ($)	With soybean peptides	Value ($)	With Tween-80	Value ($)	
Barley	6	0.4692	220.52	0.5832	274.10	0.6402	300.89	0.5988	281.43	[52]
Miscanthus	24	1.8552	871.94	2.0640	970.08	2.2824	1072.73	1.9945	937.42	[53]
Poplar	14	0.4323	203.17	0.4576	215.06	0.4672	219.59	0.5539	260.36	[54]

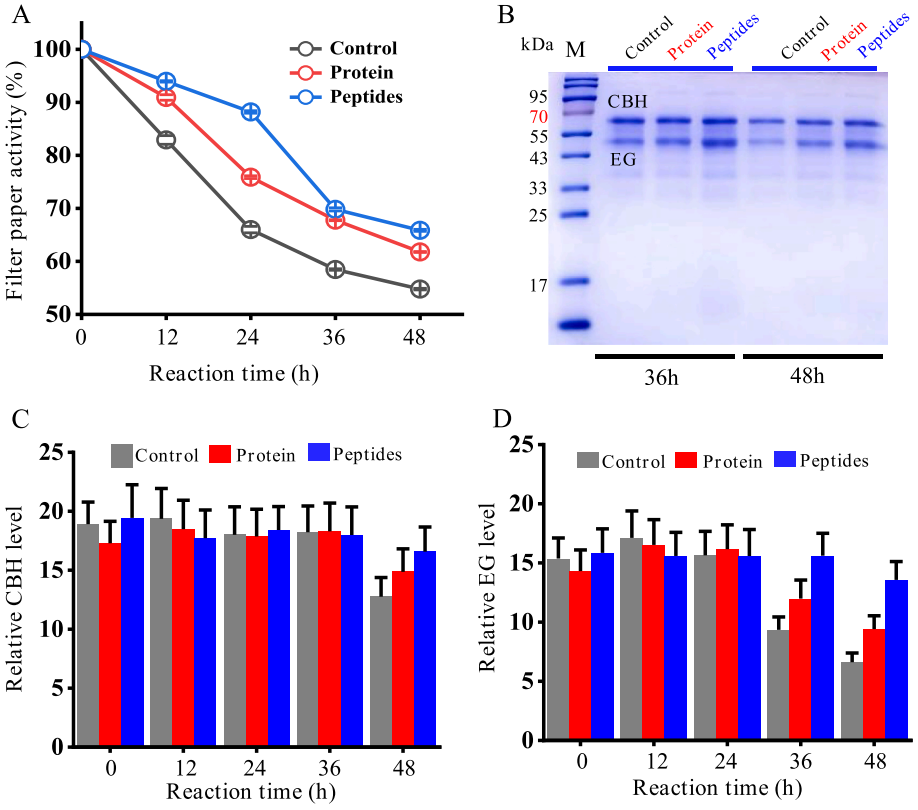

Fig. 3. Characterization of soybean protein and peptides activation for mixed-cellulases hydrolysis reaction *in vitro*. (A) Filter paper activity/FPA after incubation with mixed-cellulases co-supplied with 12 % protein or 12 % peptides; (B) SDS-PAGE profiling of mixed-cellulases separation during a time course incubation; (C, D) Semi-quantitation of CBHI and EG enzymes of mixed-cellulases during time course incubation in (B) and Fig. S4.

protein and peptides samples retained two enzymes at high levels, the peptides supply caused much higher CBHI and EG quantities than the soybean protein did after 48 h reaction, consistent with their different FPAs examined. Furthermore, all control and protein/peptides samples showed similar levels of two major enzymes during 24 h incubations, suggesting that soybean protein and peptides should also activate cellulose enzymatic hydrolyses. The results thus reveal that the soybean peptides could not only increase cellulases activity, but also play a role in preventing cellulases from self-degradation for a longer reaction.

3.4. Soybean peptides enable a broad interaction with major wall polymers for unblocking mixed-cellulases

To explore specific role of soybean peptides in biomass enzymatic saccharification, this study *in vitro* detected soybean protein and peptides interactions with three standard lignocellulose substrates (lignin, xylan, cellulose/Avicel) (Fig. 4A), which are accounting for three major wall polymers of bioenergy crops [18]. Compared to the control, about 66 % soybean protein and 69 % peptides were respectively precipitated from lignin adsorption, whereas about 49 % and 24 % of soybean protein and peptides were accounted from xylan adsorption (Fig. 4B). Notably, only peptides showed a significant precipitation with cellulose/Avicel substrate, suggesting that soybean peptides should involve in a broad interaction with three major wall polymers. Furthermore, this study attempted to observe a time course interaction among soybean protein/peptides, lignocellulose substrates and mixed-cellulases *in vitro* (Fig. 4C; Fig. S5). As total soluble proteins are mainly derived from the mixed-cellulases incubated (90 % of total), total soluble protein levels of the supernatants were detected to account for active mixed-cellulases. Among all time-course reactions examined, the soybean protein and peptides samples showed relatively higher levels of total soluble proteins than those of the control samples, revealing much more mixed-cellulases unblocked due to the soybean protein and peptides interactions with lignocelluloses. However, all peptides samples remained much higher soluble protein levels than the soybean protein did, consistent with their distinctive biomass enzymatic saccharification examined. On the other hands, total soluble proteins of the cellulose samples exhibited much more reducing levels from long time incubation among all samples detected, confirming a mixed-cellulases self-degradation during the cellulose enzymatic hydrolysis (Fig. 3). It should also interpret a relatively xylanase self-degradation in the xylan samples, owing to the mixed-cellulases containing active xylanases [40,41]. Taken all together, two modes were raised to explain characteristic soybean protein and peptides interactions with three lignocellulose

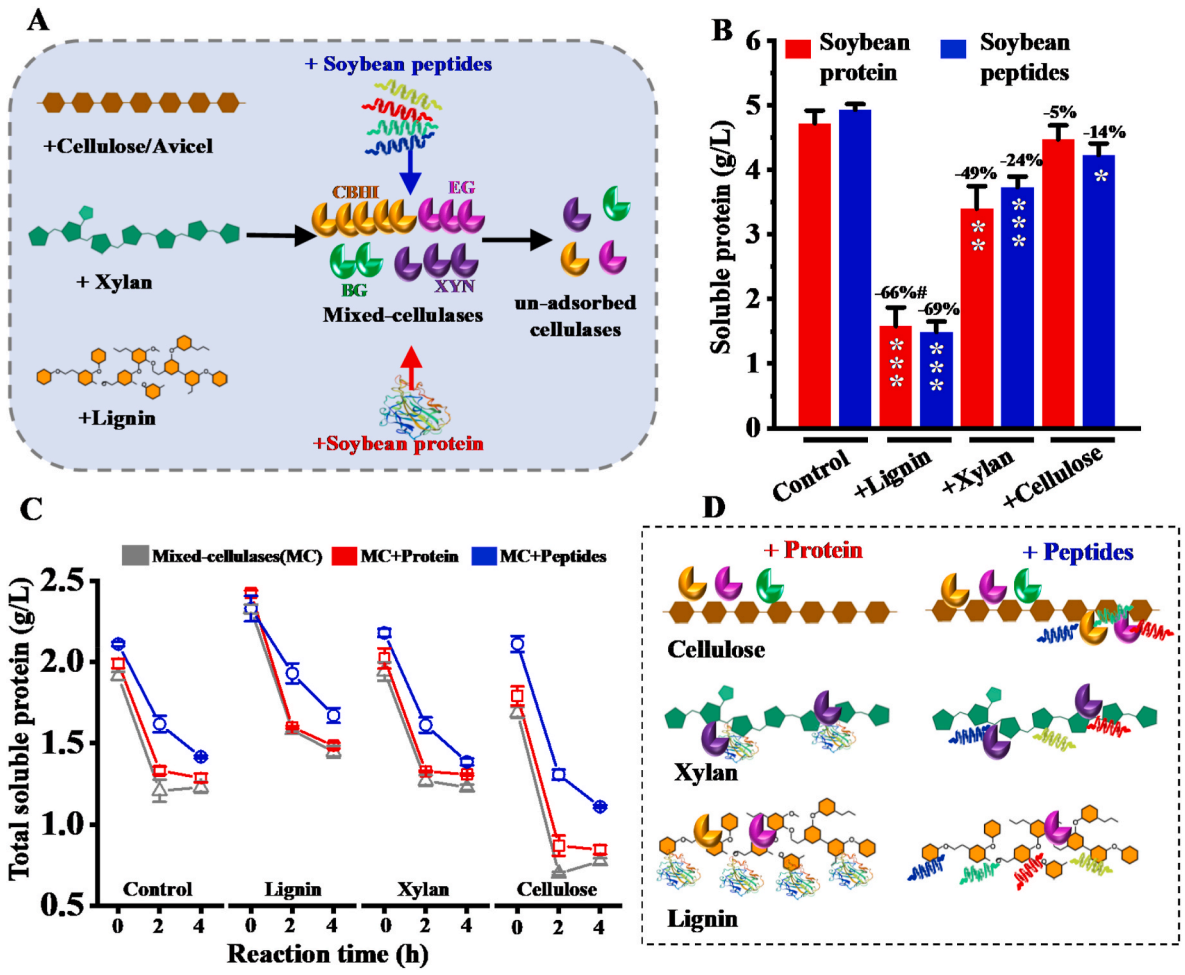

Fig. 4. Characterization of *in vitro* interactions among mixed-cellulases, soybean protein/peptides and wall polymers. (A) Experimental flow chart; (B) Assay of soluble protein in the supernatant after reaction; (C) Assay of total soluble mixed-cellulases in the supernatant after reactions for 2 h and 4 h; (D) Two modes of soybean protein and peptides interactions with lignocelluloses for distinctively enhanced enzymatic hydrolyses of cellulose and xylan; Data as means ± SD (n = 3); *, **, *** As significant different soluble protein cntents relative to the control by *t*-test at $p < 0.05$, 0.01, 0.001 levels, respectively; # As reduced percentage of protein content relative to the control.

substrates for activating mixed-cellulases (Fig. 4D). Despite of similar actions with lignin or xylan from soybean protein and peptides supplements, the peptides showed a unique interaction with cellulose substrate to facilitate mixed-cellulases accessible for cellulose hydrolysis, providing the evidence about more enhanced biomass enzymatic saccharification from the peptide supply.

3.5. Soybean peptides play biosurfactant role in biomass enzymatic saccharification

Since the soybean peptides interaction may change lignocellulose hydrophobicity, hydrogen binding capacity, and surface property [56–58], this study detected the potential surfactant roles of soybean

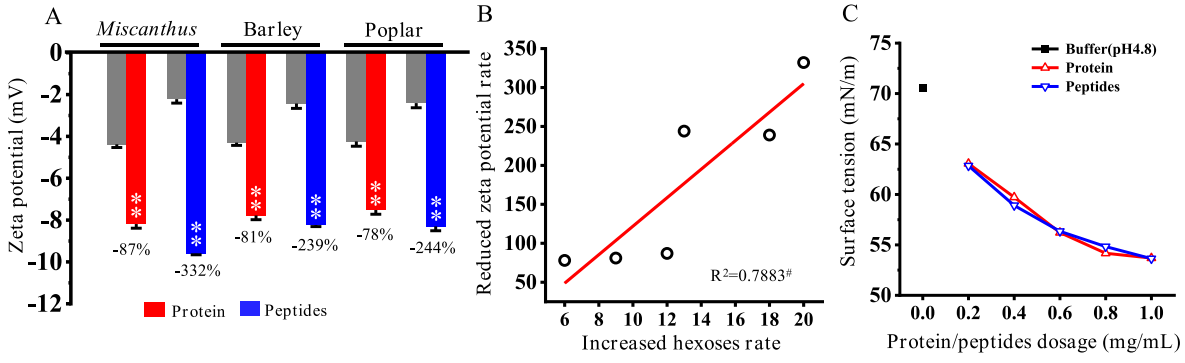

Fig. 5. Characterization of mixed-cellulases hydrolyses reactions of alkali-pretreated lignocelluloses in three bioenergy crops co-supplied with soybean protein/peptides. (A) Zeta potential assay of the supernatant after enzymatic hydrolysis; Data as means ± SD (n = 3); ** As significant difference relative to the control (without soybean protein/peptides supply) by *t*-test at $p < 0.01$ levels; (B) Correlation analysis between increased hexoses rate and reduced zeta potential rate (n = 6); (C) Surface tension assay from co-supplements of different dosages of soybean protein and peptides; # As a significant correlation at $p < 0.05$ level.

protein and peptides in biomass enzymatic saccharification of three bioenergy crops (Fig. 5). By co-supplying soybean protein or peptides into the mixed-cellulases hydrolyses with alkali-pretreated lignocellulose substrates of *Miscanthus*, barley and poplar, this study determined significantly reduced zeta potential values of all reaction solutions by 78 %–239 % at $p < 0.01$ levels (n = 3), compared to the control samples without soybean protein and peptides (Fig. 5A). Likewise, the peptides samples remained lower zeta potential values than the soybean protein samples did, consistent with their distinct enhancements of biomass enzymatic saccharification. Based on correlation analyses of all samples conducted, this study found that the reduced zeta potential rates could positively increase hexoses yield at significant level with R^2 value of 0.79 (Fig. 5B). Furthermore, this study determined the surface tensions of both soybean protein and peptides solutions at different dosages (Fig. 5C). Compared to the control, all soybean protein and peptides solutions showed much reduced surface tension values, which should be accountable for their improved biosurfactant property. Meanwhile, relatively lower surface tensions were detected in supernatants of the enzymatic saccharification of *Miscanthus* alkali-pretreated lignocelluloses co-supplied with soybean protein and peptides (Table S3; Fig. 1E). As plant proteins have been characterized as effective biosurfactants [12,18], the results reveal that soybean peptides could even act as a better biosurfactant for enhancing biomass enzymatic saccharification.

3.6. Mechanisms of distinct soybean protein and peptides enhancements for biomass enzymatic saccharification and bioethanol production

Based on all findings achieved in this study, a mechanism model was proposed to illuminate how soybean protein and peptides supplements could distinctively enhance biomass enzymatic saccharification and bioethanol production in bioenergy crops (Fig. 6). As three representative bioenergy crops provide distinct lignocelluloses substrates, the alkali pretreatments caused largely varied extractions of lignin and hemicellulose. Three major polymers (cellulose, hemicellulose, lignin) of alkali-pretreated lignocelluloses were thus accessible to interact with soybean peptides, whereas the soybean protein only interacted with hemicellulose and lignin. Such broad interactions of soybean peptides could not only efficiently block lignin adsorption with mixed-cellulases, but also effectively retain the mixed-cellulases to attack and digest cellulose, which should cause an integrative enhancement of biomass enzymatic saccharification for relatively higher bioethanol production in bioenergy crops. This may also explain why the same dosage of soybean protein caused relatively less enhancement of biomass enzymatic saccharification than the peptides did, due to its non-interaction with cellulose microfibrils. Nevertheless, it would be interesting to explore how soybean peptides enable interaction with cellulose microfibrils for improving lignocellulose enzymatic hydrolysis in future study.

4. Conclusion

By employing distinct lignocellulose substrates of three representative bioenergy crops, this study demonstrates that soybean peptides supply could exceptionally enhance biomass enzymatic saccharification to achieve maximum bioethanol production compared to the soybean protein and Tween-80 as previously reported. The soybean peptides enable a universal interaction with lignin and xylan to prevent mixed-cellulases from any non-specific adsorption as the soybean protein and Tween-80 do. However, unlike the soybean protein and Tween-80, the soybean peptides are identified with a unique interaction with cellulose microfibrils to facilitate cellulases accessible for cellulose hydrolysis. A novel mechanism model is thus raised to elucidate how soybean peptides cause dual enhancements of hexoses and bioethanol production via exceptional interactions with three major wall polymers, providing an advanced biotechnology for biomass saccharification and bioethanol production in bioenergy crops.

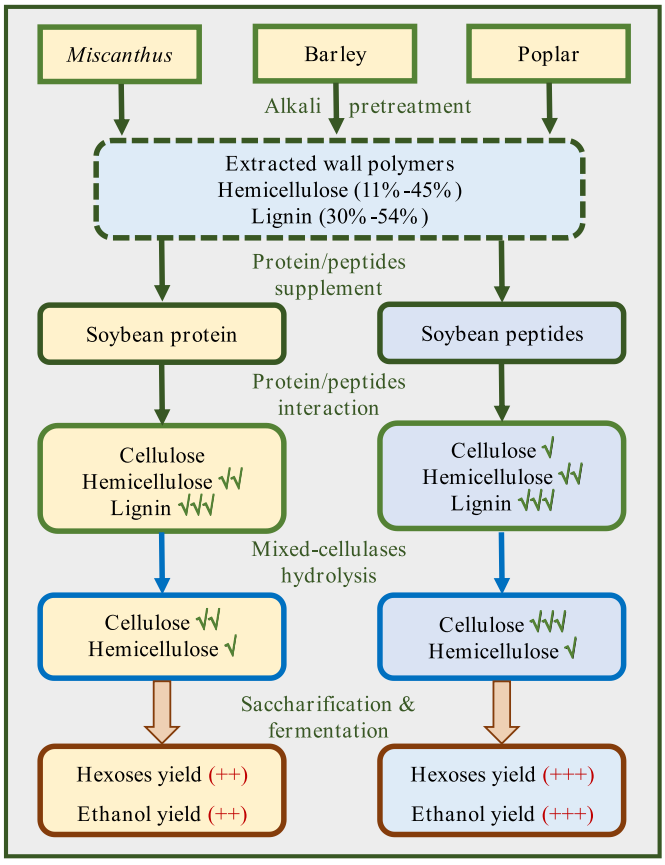

Fig. 6. A mechanism model about how soybean protein and its peptides supplements distinctively enhance biomass enzymatic saccharification and bioethanol conversion in three bioenergy crops. √ As wall polymer interaction frequency or cellulose/hemicellulose hydrolysis intensity; (+) As enhanced degree.

CRediT authorship contribution statement

Shijie Lu: Writing – original draft, Methodology, Investigation, Formal analysis. **Le Sun:** Writing – review & editing, Investigation, Formal analysis. **Yixiang Wang:** Methodology, Investigation, Formal analysis. **Hao Peng:** Methodology, Investigation, Formal analysis. **Boyang He:** Methodology, Investigation, Formal analysis. **Xiaoling Yuan:** Methodology, Investigation, Formal analysis. **Yanting Wang:** Validation, Supervision, Funding acquisition. **Tao Xia:** Validation, Supervision, Funding acquisition. **Liangcai Peng:** Validation, Supervision, Funding acquisition. **Peng Liu:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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