Preparation of starch derivatives bearing urea groups and the evaluation of antioxidant, antifungal, and antibacterial activities

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Abstract

Several starch derivatives bearing urea groups (TUCAST, MTUCAST, and PTUCAST) were successfully designed and synthesized. FT-IR, 1H NMR spectroscopy, and elemental analysis were carried out to identify the structural characteristics of starch derivatives. The antioxidant activity of starch derivatives was tested by superoxide-radical scavenging and hydroxyl-radical scavenging assays. Their antifungal activity against Fusarium oxysporum f. sp. cucumerinum Owen, Phomopsis asparagus, and Fusarium oxysporum f. sp. niveum was estimated by hyphal measurement. Furthermore, the antibacterial activity against Escherichia coli and Staphylococcus aureus was evaluated by optical density method. Compared to starch, the products bearing urea groups showed enhanced antifungal action as well as an excellent antioxidant and antibacterial activities. Their bioactivities decreased roughly in the order of PTUCAST > MTUCAST > TUCAST > CAST > starch, which matched with the electron-withdrawing property of the different substituted groups of urea. Besides, the cytotoxic activity of starch and synthesized derivatives against L929 cells was evaluated using CCK-8 assay in vitro and all samples showed weak cytotoxicity. The results suggested that these novel starch derivatives possessing satisfactory bioactivities could be widely used in the food industry.

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1. Introduction

Starch is the most abundant storage polysaccharide existing in a large variety of plants, such as cereals, legumes, and tubers [1–3]. Besides serving as a food source, starch has vast applications in fields of cosmetics, textiles, and pharmaceutical industries as well as some environmentally correct materials, including bags, cups, cutlery, and food packaging due to the benefits of renewability, biodegradability, and biocompatibility [4–7]. However, one of the enormous challenges to using pristine starch in some applications is the lower bioactivities on account of the shortage of active functional groups such as amino, sulfate ester, and carboxyl groups [2,8]. For this reason, chemical modification was proposed which could drastically enhance the original bioactivities of starch and generate new functionalities by introducing functional groups into starch backbones [9–11].

In recent years, our group has devoted extensive efforts on the preparation of functional starch derivatives by chemical modification, in order to increase its bioactivities. For instance, several 1,2,3-triazole-functionalized starch derivatives containing hydroxalkyl with different chain were synthesized by ‘click chemistry’ and had exhibited effective antioxidant activity against hydroxyl-radical, DPPH-radical, and superoxide-radical. Their scavenging ability increased with increasing side-chain length [8]. The antifungal property of quaternized 1,2,3-triazole-functionalized starch derivatives was also studied. The results indicated that the synthesized 1,2,3-triazolium-functionalized starch derivatives clearly showed stronger antifungal activity and the 1,2,3-triazolium moieties should be excellent antifungal function groups [9]. Moreover, the preparation, characterization, and antifungal property of starch derivative bearing 1,2,3-triazole and pyridine as well as starch
derivative bearing 1,2,3-triazolium and pyridinium were investigated. The synthesized starch derivative bearing 1,2,3-triazolium and pyridinium showed more significant antifungal activity than starch derivative with 1,2,3-triazole and pyridine and starch [2].

In addition to the ‘click reaction’, we also deeply researched the synthesis of starch derivatives with high bioactivity via chloroacetyl starch intermediates. For example, several novel antifungal starch derivatives bearing quaternary phosphonium groups were successfully prepared and their antifungal activity could be influenced by the electron-withdrawing ability of different substituted groups in quaternary phosphonium salts. Additionally, synthetic starch derivatives bearing Schiff bases had also attracted interest as antibacterial agents according to our previous report [12].

Hence, chemical modification is a promising handling for high value utilization of starch.

Thiadiazole, with a bioactive -N=C-S- grouping, has played an important role in research fields of antimicrobial [13], anticancer [14], anticonvulsant [15], anti-inflammatory [16], and antipsychotic [17] agents. For example, Abdel-Wahab et al. had synthesized a new series of 1,3,4-thiadiazole derivatives of 5-(benzofuran-2-yl)-1-phenylpyrazole moiety and evaluated them for their antibacterial activity which was found to possess significant activity against E. coli and C. albicans [18]. Lavanya et al. had described an efficient and benign synthesis of 1,3,4-thiadiazole systems containing pyrazoles and pyrroles and this novel 1,3,4-thiadiazole derivatives emerged the potential of being anti-inflammatory agents [19]. In addition, urea group has been frequently found as a key structural motif in antifungal agents. Past researches have demonstrated the important role of urea groups in increasing the bioactivity of some polysaccharides [20,21]. Based on the above analyses, we planned to synthesize several novel urea groups containing thiadiazole. This combination aims to graft some active groups onto starch to enhance the bioactivities.

In continuation of our successful attempts in search for biologically active polysaccharide derivatives, in present study we report the synthesis, characterization, antioxidant, antifungal, and antibacterial screening of some starch derivatives bearing thiadiazole-structured urea groups. Since polysaccharides with chloride acetyl group could attack pyridine to give N-alkypyridinium salts, 6-O-chloroacetylated starch (CAST) was synthesized by the reaction of starch with chloroacetyl chloride [2]. The synthesized starch derivative bearing 1,2,3-triazolium and pyridinium were investigated [2].

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2. Materials and methods

2.1. Materials

Starch with a molecular weight of 9.8 × 10⁶ Da was supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The amylose content of the starch reported by the company was 20–25%. Chloroacetyl chloride, nicotinoyl chloride hydrochloride, 2-amino-1,3,4-thiadiazole, and 2-amino-5-methyl-1,3,4-thiadiazole were purchased from the Sigma-Aldrich Chemical Corp (Shanghai, China). The other reagents, such as sodium azide, N-Methyl pyrrolidone (NMP), N, N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetone, ethanol, methylbenzene, etc. were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and used as received.

2.2. Analytical methods

2.2.1. Fourier transform infrared (FT-IR) spectroscopy

The infrared spectra of the samples were obtained using a Jasco-4100 FT-IR spectrometer (Japan, provided by JASCO Co., Ltd., Shanghai, China) in the range of 4000–400 cm⁻¹ with a resolution of 4.0 cm⁻¹. The tested samples were lamellated with KBr in the weight ratio 1/100 for observations.

2.2.2. Nuclear magnetic resonance (NMR) spectroscopy

The ¹H NMR spectra of chitosan derivatives were recorded on a Bruker AVIII-500 Spectrometer (500 MHz, Switzerland, provided by Bruker Tech. and Serv. Co., Ltd., Beijing, China) at 25 °C. The samples were dissolved in DMSO for analysis.

The degrees of substitution (DS) of CAST were estimated from the ¹H NMR spectrum by using relative change in the ratio of integral of the hydrogen atom bonded to carbon 1 of the glucopyranose ring and hydrogen atoms corresponding to chloroacetyl group [22]. The DS of CAST were calculated according to the following equation:

\[ DS(\%) = \frac{I_{CAST}}{I_{CH2Cl}} \times 100 \]

where \( I_{CAST} \) is the integral of the hydrogen atoms on chloroacetyl group (4.4–4.5 ppm); \( I_{CH2Cl} \) is the integral of the hydrogen atom bonded to carbon 1 of the glucopyranose ring (5.2–6.0 ppm); 2 is the number of protons in methylene (-CH₂Cl). The results are shown in Fig. 3.

2.2.3. Elemental analysis

The elemental analyses (C, H, N, and S) were performed on a Vario Micro Elemental Analyzer (Elementar, Berlin, Germany). The carbon nitrogen ratios were used to evaluate the degree of substitution in starch derivatives and the DS were calculated by the following equations:

\[ DS = \frac{n_1 \times M_C + n_2 \times M_C \times DS_{CAST}}{m \times M_N + W_C/N - n_3 \times M_C} \]

where DS represents the urea groups in starch derivatives (TUCAST, MTUCAST, or PTUCAST); DSCAST represents the chloroacetyl group in starch derivative (CAST), which is estimated on the basis of the integral values in ¹H NMR spectrum, \( DS_{CAST} = 0.82; M_C \) and \( M_N \) are the molar mass of carbon and nitrogen, \( M_C = 12, M_N = 14; n_1, n_2, \) and \( n_3 \) are the number of carbon of starch, chloroacetyl group, and urea group, \( n_1 = 6, n_2 = 2, TUCAST: n_3 = 8, MTUCAST: n_3 = 9, PTUCAST: n_3 = 14; n \) is the number of protons in urea group, \( n = 5; W_C/N \) represents the mass ratio between carbon and nitrogen in starch derivatives (Scheme 1).

2.3. Synthesis of starch derivatives

2.3.1. Synthesis of urea groups

Firstly, nicotinyl chloride hydrochloride (20 mmol) was dispersed equally in 15 mL of acetone and stirred at 0 °C. The mixture was then added dropwise to the aqueous solution of sodium azide (3.9 g NaN₃ dissolving in 12 mL of deionized water). The reaction mixture was stirred for 3 h under the condition of ice bath. After the reaction was finished, the solution had been stratified and the lower layer was removed. The remaining solution was poured into 10 mL of methylbenzene solvent at 60 °C and stirred for 2–3 h.
Then, the mixture was cooled and some pink crystals were precipitated out. The precipitate was filtered and pyridine-3-isocyanate was obtained.

Finally, pyridine-3-isocyanate (30 mmol) and 2-amino-1,3,4-thiadiazole, 2-amino-5-methyl-1,3,4-thiadiazole, or 2-amino-5-phenyl-1,3,4-thiadiazole were added to methylbenzene (20 mL) in a 50 mL flask. The mixture was stirred for 24 h at 60 °C, then a large amount of solid formed and was filtered, which was further purified by crystallization from the solvent that the ratio of water and ethanol was 1:1 and several urea groups were synthesized.

2.3.2. Synthesis of chloracetyl starch (CAST)

CAST was synthesized according to the following method: firstly, starch (1.62 g, 10 mmol) was dispersed in 100 mL of H₂O at room temperature (r.t.). Then, 20 mmol chloracetyl chloride was added slowly. After continuous stirring for 12 h at r.t., the solution was concentrated under the condition of reduced pressure at 70 °C. The concentrated solution was poured into 200 mL of alcohol to obtain some precipitates. The precipitate was filtered and washed with ethanol for three times. Finally, CAST was achieved after vacuum freeze-drying for 24 h.

2.3.3. Synthesis of starch derivatives (TUCAST, MTUCAST, and PTUCAST)

1 mmol CAST and 3 mmol urea groups dissolved in 20 mL of N, N-dimethylformamide (DMF) were stirred for 24 h at 60 °C. The solution was precipitated in excess acetone. Then the precipitate was filtered and washed with ethanol for three times. Finally, the starch derivatives (TUCAST, MTUCAST, and PTUCAST) were obtained after drying at 60 °C for 6 h.

2.4. Antioxidant assays

2.4.1. Superoxide-radical scavenging activity assay

The superoxide-radical scavenging ability was assessed following the model of Tan’s methods [22]. The reaction mixture, involving test samples (5 mg/mL, 0.06, 0.12, 0.24, 0.48, and 0.96 mL), phenazine methosulfate (PMS, 30 μM), nitro blue tetrazolium (NBT, 72 μM), and nicotinamide adenine dinucleotide reduced (NADH, 338 μM) in Tris–HCl buffer (16 mM, pH 8.0), was incubated 5 min at r.t.. The absorbance was measured at 560 nm. Three replicates for each sample were tested and the superoxide-radical scavenging effect was calculated according to the following equation:

\[
\text{Scavenging effect} \% = \left(1 - \frac{A_{\text{sample 560 nm}} - A_{\text{control 560 nm}}}{A_{\text{blank 560 nm}}}\right) \times 100
\]

where \(A_{\text{sample 560 nm}}\) is the absorbance of the samples, \(A_{\text{control 560 nm}}\) is the absorbance of the control (NADH was substituted with distilled water), and \(A_{\text{blank 560 nm}}\) is the absorbance of the blank (samples were substituted with distilled water).

2.4.2. Hydroxyl-radical scavenging activity assay

The test of hydroxyl-radical scavenging ability was carried out according to Li’s methods with minor modification [23]. The reaction mixture, containing testing samples of starch or starch derivatives (10 mg/mL, 0.045, 0.09, 0.18, 0.36, and 0.72 mL), safranine O (0.23 μM), EDTA-Fe²⁺ (220 μM), and H₂O₂ (60 μM) in potassium phosphate buffer (150 mM, pH 7.4), was incubated for 30 min at 37 °C. The absorbance of the mixture was measured at 520 nm against blank. Three replicates for each sample were tested and the hydroxyl-radical scavenging effect was calculated according to the following equation:

\[
\text{Scavenging effect} \% = \left(1 - \frac{A_{\text{sample 520 nm}} - A_{\text{blank 520 nm}}}{A_{\text{control 520 nm}} - A_{\text{blank 520 nm}}}\right) \times 100
\]

where \(A_{\text{sample 520 nm}}\) is the absorbance of the samples, \(A_{\text{control 520 nm}}\) is the absorbance of the control (H₂O₂ was substituted with potassium phosphate buffer), and \(A_{\text{blank 520 nm}}\) is the absorbance of the blank (samples were substituted with distilled water).

2.5. Antifungal assay

The antifungal ability was carried out by the method of hyphal measurement [9]. Briefly, the stock solutions of starch and derivatives were prepared with a concentration of 6 mg/mL. Then, each
sample solution was added to sterilized potato dextrose agar (PDA) medium to obtain final concentrations of 0.1, 0.5 and 1.0 mg/mL. The culture media containing samples were poured into Petri dishes (7 cm). After solidification, 5.0 mm diameter of fungi mycelium was transferred to the test plate and incubated at 27 °C for 2–3 days. When the mycelia of fungi reached the edges of the control plate (without the presence of samples), the inhibition indices of all samples were calculated as follows:

Antifungal index (%) = \( \frac{1 - D_a/D_b}{} \times 100 \)

where \( D_a \) is the diameter of the growth zone in the test plates and \( D_b \) is the diameter of the growth zone in the control plate.

2.6. Antibacterial assay

The antibacterial property of starch and starch derivatives against \( \text{E. coli} \) and \( \text{S. aureus} \) was tested by optical density method. \( \text{E. coli} \) and \( \text{S. aureus} \) were incubated in nutrient broth (containing 10 g peptone, 5 g beef extracts, 10 g NaCl, and 1 L deionized water) at 37 °C for 24 h. Both bacterial species at the exponential growth phase were harvested in this way. Then, the culture was diluted to get a bacterial suspension containing \( 10^5 \)–\( 10^6 \) cells/mL. Samples of phase were harvested in this way. Then, the culture was diluted to \( 10^5 \)–\( 10^6 \) cells/mL. Samples of phase were harvested in this way. Then, the culture was diluted to

\[ C176 \times 10 \text{g peptone, 5 g beef extracts, 10 g NaCl, and 1 L deionized water} \)

\[ E. coli \]

\[ \text{S. aureus} \]

\[ \text{A} \]

\[ \text{D} \]

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\[ 1274 \]

\[ 3.1. \text{Chemical synthesis and characterization} \]

Firstly, CAST was synthesized by the reaction of starch with chloroacetyl chloride, as chloroacetyl chloride is an attractive bridge group to connect starch molecule and urea groups bearing pyridine ring. Compared with the characteristic absorbance bands of starch at 3428 cm\(^{-1}\) and 1427 cm\(^{-1}\) (O–H stretching vibrations), 2927 cm\(^{-1}\) and 1373 cm\(^{-1}\) (C–H stretching and deformation vibration), and 1200–990 cm\(^{-1}\) (C–C and C–O stretching vibrations) [24,25], the FT-IR spectrum of CAST shows characteristic peaks at 1747 cm\(^{-1}\) and 786 cm\(^{-1}\) [12,21], which can be attributed to the vibrations of C–O and C–Cl in chloracetyl group, respectively. Accordingly, the signals of the methylene protons of -COCH\(_2\)Cl group appear at 4.4 pm in the \(^1\)H NMR spectrum of CAST [20,21], which further demonstrate the successful introduction of chloracetyl group. Because of the absence of nitrogen from the chloro acetyl group, the DS of CAST was evaluated based on the integral values in the \(^1\)H NMR spectrum according to the earlier report. The DS of CAST is 0.82, which means that 82% of the hydroxyl groups of starch at C-6 had reacted with chloroacetyl chloride.

Then, several urea groups were obtained following the reaction of pyridine-3-isocyanate and amino-thiadiazole. Because polysaccharides with chloride acetyl groups can attack pyridine to form N-alkylpyridinium salts, these active groups were introduced into CAST and the final products (TUCAST, MTUCAST, and PTUCAST).

2.7. Cytotoxicity assay

The cytotoxicity of starch and synthesized starch derivatives on L929 cells at different concentrations (1.0, 10.0, 100.0, 500.0, and 1000.0 \( \mu g/mL \)) was determined by CCK-8 assay in vitro. L929 cells were cultured at 37 °C in RPMI medium (containing 1% mixture of penicillin & streptomycin and 10% fetal calf serum). The cells were seeded on 96-well flat-bottom culture plates at a density of 1.0 \( \times 10^5 \) cells and incubated under 5% CO\(_2\) atmosphere. After 24 h of cell attachment, the cells with different final concentrations were introduced to cells, separately. Next, the cells were cultured for 24 h. Afterward, 10 \( \mu l \) of CCK-8 solution was added in each well and incubated for another 24 h at 37 °C. The absorbance at 450 nm was measured. Cell viability was recorded according to the following formula:

Cell viability (%) = \( \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{negative}} - A_{\text{blank}}} \times 100 \)

where \( A_{\text{sample}} \) is the absorbance of the samples (containing cells, CCK-8 solution, and sample solution), \( A_{\text{blank}} \) is the absorbance of the blank (containing RPMI medium and CCK-8 solution), and \( A_{\text{negative}} \) is the absorbance of the negative (containing cells and CCK-8 solution).

2.8. Statistical analysis

All the data related to antioxidant activity, antifungal activity, antibacterial activity, and cytotoxicity assay were illustrated as

mean \( \pm \) standard deviation (SD), \( n = 3 \). Significant difference analysis was determined using Scheffe’s multiple range test. The significant differences were defined at \( p < 0.05 \).

3. Results and discussion

3.1. Chemical synthesis and characterization

We verified the products synthesized at each step by FTIR (Fig. 1), \(^1\)H NMR (Fig. 1), and elemental analysis (Table. 1).

Firstly, CAST was synthesized by the reaction of starch with chloroacetyl chloride, as chloroacetyl chloride is an attractive bridge group to connect starch molecule and urea groups bearing pyridine ring. Compared with the characteristic absorbance bands of starch at 3428 cm\(^{-1}\) and 1427 cm\(^{-1}\) (O–H stretching vibrations), 2927 cm\(^{-1}\) and 1373 cm\(^{-1}\) (C–H stretching and deformation vibration), and 1200–990 cm\(^{-1}\) (C–C and C–O stretching vibrations) [24,25], the FT-IR spectrum of CAST shows characteristic peaks at 1747 cm\(^{-1}\) and 786 cm\(^{-1}\) [12,21], which can be attributed to the vibrations of C–O and C–Cl in chloracetyl group, respectively. Accordingly, the signals of the methylene protons of -COCH\(_2\)Cl group appear at 4.4 pm in the \(^1\)H NMR spectrum of CAST [20,21], which further demonstrate the successful introduction of chloracetyl group. Because of the absence of nitrogen from the chloro acetyl group, the DS of CAST was evaluated based on the integral values in the \(^1\)H NMR spectrum according to the earlier report. The DS of CAST is 0.82, which means that 82% of the hydroxyl groups of starch at C-6 had reacted with chloroacetyl chloride.

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were synthesized in this way. With taking advantage of the principle of active superposition, these compounds were expected to possess improved bioactivities. Meanwhile, the water solubility of starch derivatives should be enhanced since the formation of N-alkyppyridinium salts.

In the FT-IR spectra of TUCAST, MTUCAST, and PTUCAST, the vibration peaks at 786 cm$^{-1}$ confirm that the existence of C–Cl becomes weaker and new peaks appear at about 1550 cm$^{-1}$, 1510 cm$^{-1}$, and 670 cm$^{-1}$, indicating that C–Cl bonds were replaced by some new groups bearing aromatic rings. Exactly, the peaks at 1550 cm$^{-1}$ and 1510 cm$^{-1}$ are the characteristic signals of pyridine [22,26] as well as the peak at 670 cm$^{-1}$ is the characteristic signal of thiadiazole [27,28]. Moreover, the FT-IR spectrum of PTUCAST shows absorption at 771 cm$^{-1}$, which can be assigned to the typical absorption of benzene. Therefore, we can preliminarily conclude that urea groups were introduced into CAST. As to the $^1$H NMR spectra of TUCAST, MTUCAST, and PTUCAST, the peak of –COCH$_2$Cl at 4.4 ppm get weaker and new chemical shifts appear at 7.0–9.5 ppm, confirming the existence of urea groups containing pyridine and thiadiazole [29,30]. The peak at 9.4 ppm can be assigned to the protons on –NH of the urea groups. Particularly, the specific positions of the hydrogen protons of urea groups are marked in Fig. 2 (TUCAST: peaks at 8.0–9.2 ppm and 7.75 ppm are assigned to the protons on pyridine and thiadiazole, respectively. MTUCAST: peaks at 7.8–8.7 ppm and 2.4 ppm are assigned to the protons on pyridine and methyl, respectively. PTUCAST: peaks at 7.0–7.8 ppm and 7.8–8.7 ppm are assigned to the protons on benzene and pyridine, respectively.). These data further confirmed the successful syntheses of starch derivatives bearing urea groups. The DS with urea groups for starch derivatives were estimated by elemental analysis in Table 1. The DS of TUCAST and MTUCAST are about 0.3, while the DS value of PTUCAST is only 0.22. The lower DS of PTUCAST may be resulted from the large steric hindrance of benzene ring.

### 3.2. Antioxidant activity

Free radicals can cause structural damage to cells, and such damage is thought to be one of the primary causes of many lifestyle-related diseases. The application of antioxidants is a useful way to eliminate free radicals. In this paper, several starch derivatives with high antioxidant activity were synthesized and their superoxide-radical scavenging activity (a) and hydroxyl-radical scavenging activity (b) are shown in Fig. 4.

To verify whether the antioxidant activity of starch derivatives was improved by grafting with urea groups, their antioxidant activity was evaluated by superoxide radical scavenging assay and hydroxyl radical scavenging assay. As shown in Fig. 4, the superoxide radical scavenging activity and hydroxyl radical scavenging activity of all samples are concentration-dependent. Compared with starch and CAST, the final starch derivatives bearing urea groups show higher antioxidant activity. Thus, this improved antioxidant ability is mainly attributed to the function of urea groups. In superoxide radical scavenging assay, TUCAST, MTUCAST, and PTUCAST have almost the same antioxidant capacity when the concentration is below 0.4 mg/mL. At high concentrations, their

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Yields (%)</th>
<th>Elemental analyses (%)</th>
<th>Degrees of Substitution</th>
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<tr>
<td></td>
<td></td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>TUCAST</td>
<td>63.2</td>
<td>39.615</td>
<td>7.455</td>
</tr>
<tr>
<td>MTUCAST</td>
<td>61.2</td>
<td>29.291</td>
<td>5.208</td>
</tr>
<tr>
<td>PTUCAST</td>
<td>64.8</td>
<td>40.583</td>
<td>4.786</td>
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Fig. 2. $^1$H NMR spectra of starch and starch derivatives.
scavenging rates show a certain rule, that is PTUCAST > MTUCAST > TUCAST. In hydroxyl radical scavenging assay, the scavenging capacity of TUCAST is always lower than that of MTUCAST and PTUCAST at the test concentration. As for MTUCAST and PTUCAST, when the concentration is lower than 0.6 mg/mL, the antioxidant capacity of MTUCAST is stronger than that of PTUCAST. However, when the concentration is higher than 0.6 mg/mL, the antioxidant capacity of PTUCAST gradually exceeds that of MTUCAST.

3.3. Antifungal activity

Plant pathogen fungi is a notorious agricultural microorganism that can cause severe disease and enormous economic losses worldwide. Nowadays, chemical pesticides are widely applied to prevent various agricultural diseases due to their high efficiency. However, the widely used chemical pesticides are causing great harm to the environment and human health. Hence, screening
Fig. 6. The antibacterial activity of starch and starch derivatives against *E. coli* at 8 h (a), *E. coli* at 16 h (b), *S. aureus* at 8 h (c), and *S. aureus* at 16 h (d).

Fig. 7. The cytotoxicity of starch and starch derivatives on L929 cells.
for novel antifungal agents that are eco-friendly is highly desired. In this paper, several biodegradable starch derivatives were synthesized and their antifungal activity against F. oxysporum f. sp. cucumeribrium Owen, P. asparagus, and F. oxysporum f. sp. niveum was studied. The test results are shown in Fig. 5.

As seen in Fig. 5, the antifungal activity of all samples is susceptible to the sample concentration and there is a positive correlation between them. Meanwhile, the antifungal ability of the final starch derivatives (TUCAST, MTUCAST, and PTUCAST) is stronger than that of starch and CAST. For example, the inhibitory indices of TUCAST, MTUCAST, and PTUCAST against F. oxysporum f. sp. cucumeribrium Owen are 40.48, 45.77, and 56.11%, while that of starch and CAST are only 7.11 and 18.67%. This phenomenon could also be observed in inhibiting the growth of other fungi. Thus it can be seen that the enhanced antifungal activity of starch derivatives may mainly benefit from the urea groups. In addition, it seems that the antifungal activity of PTUCAST is stronger than that of MTUCAST, and the activity of MTUCAST is stronger than that of TUCAST. This antifungal rule is in accord with the order of the electronacceptivity of urea groups (phenyl > methyl > hydrogen) in starch derivatives. For instance, the inhibitory indices of TUCAST, MTUCAST, and TUCAST against F. oxysporum f. sp. cucumeribrium Owen are 40.48, 45.77, and 56.11%, respectively. Exceptionally, when inhibiting the growth of P. asparagus, the ability of PTUCAST is slightly weaker than that of MTUCAST. Maybe the lower DS for PTUCAST affects its antifungal activity.

3.4. Antibacterial activity

Microbial contamination is a serious problem in the field of food, which could affect the quality of food and shorten its shelf life. At present, synthetic antibacterial agents have been widely used in the food industry. However, the safety of synthetic antimicrobials has become a major concern for consumers. Thus, it is urgent to find alternative riskless antibacterial agents to prevent bacterial contamination in the food industry. In this study, the antibacterial activity of starch and starch derivatives against E. coli and S. aureus was studied and the results are shown in Fig. 6.

According to Fig. 6, several conclusions can be gained about the antibacterial activity of starch and starch derivatives: firstly, the antibacterial property of all samples is concentration-dependent and the inhibitory rates increase with concentration. Secondly, compared with starch and CAST, the final products bearing urea groups have a better inhibitory effect on E. coli and S. aureus. For example, the inhibitory rates of starch, CAST, TUCAST, MTUCAST, and PTUCAST against S. aureus are 10.30%, 10.42%, 42.66%, 73.43%, and 86.01% at 1.0 mg/mL. Thirdly, when the culture time was 16 h, the antibacterial ability of some samples was reduced. This trend is more obvious when tested against S. aureus. Moreover, the inhibitory rule of all samples on E. coli is MTUCAST > TUCAST > CAST > starchy, while the inhibitory rule on S. aureus is PTUCAST > MTUCAST > TUCAST > CAST > starchy. Maybe the electron-withdrawing property of urea groups and DS are both key factors affecting the antibacterial ability.

According to the analysis of bioactive rules of the starch derivatives, there should be two reasons for explaining it: (i) the final starch derivatives with strong electron-withdrawing property can react with the negatively charged cell wall of microorganisms, which will lead to a significant change of the membranes structure of cells. This change may cause the leakage of intracellular electrolytes and proteinaceous compounds and prevent nutrients from entering the cells, which will eventually lead to cell death [31,32]. These electrophilic derivatives could also attract more single electron of free radicals to inhibit the free radical chain reaction [33,34]. Hence, the starch derivatives with stronger electron absorption ability possess better antifungal, antibacterial, and antioxidant activities. (ii) the substitution degree is another factor affecting the biological activity [35]. Generally, the higher the DS as well as more functional groups, the stronger the bioactivity. Therefore, even if MTUCAST has weaker electronacceptivity compared with PTUCAST, it could show a stronger biological activity sometimes because of the higher degree of substitution. Future research on the mechanism of biological activity of these starch derivatives will be carried out.

3.5. Cytotoxicity analysis

Samples that can support cell activity to promote its growth are regarded as biocompatible. In order to explore the biocompatibility of starch and starch derivatives, the cell viability of L929 cells by CCK-8 assay was studied and the results are shown in Fig. 7. As seen, the cell viabilities of L929 cells treated with starch are >90% at all tested concentrations. However, the cytotoxic effect of CAST is significantly higher than starch and starch derivatives bearing urea groups. When the concentration is 1000 μg/mL, the cell viability is 32.57%, which means that it is toxic to normal cells. As to TUCAST, MTUCAST, and PTUCAST, their cytotoxic effects are similar to those of pristine starch. Hence, starch derivatives could be considered to have good biocompatibility.

4. Conclusions

In present study, a new class of starch derivatives was synthesized through two step reaction. Their structural characterization was confirmed using FT-IR and 1H NMR spectroscopy. Their antifungal activity against three common plant-threatening fungi was estimated by hyphal measurement in vitro. The antibacterial property against E. coli and S. aureus was investigated by optical density method. Meanwhile, two antioxidant traits, including superoxide radical scavenging activity and hydroxyl radical scavenging activity were tested. The activity test results showed that the starch derivatives bearing urea groups exhibited enhanced antifungal activity as well as strong antibacterial property and scavenging effect. The key role of urea groups in enhancing the bioactivity of starch is obvious. Moreover, their bioactivity could be influenced by the electron-withdrawing capacity and substitution degree. The starch derivatives with higher electron-withdrawing capacity and substitution degree possess better bioactivity. In addition, the cytotoxic effect of starch derivatives was assessed by CCK-8 method. The cytotoxic results indicated that the synthetic starch derivatives bearing urea groups showed decreased cytotoxic effect. Therefore, these new types of urea-functionalized starch derivatives represent a novel class of polymers with high antibacterial and antioxidant activities, which could be used for fruits, meat, and dairy products as food packaging. Of course, the research on the application of these starch derivatives to starch-based films in food packaging should be further investigated.

Declaration of competing interest

The authors have declared no conflicts of interest.

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