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In vitro antioxidant activity of liquor and semi-purified fractions from fermented squid pen biowaste by *Serratia ureilytica* TKU013

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ABSTRACT

Antioxidative activities were found in the culture supernatant of *Serratia ureilytica* TKU013 with squid pen as the sole carbon/nitrogen source. The 4th day supernatant showed the strongest antioxidant activities and the highest total phenolic content. The supernatant was further purified by liquid–liquid partition, and it was found that the ethyl acetate-soluble (EA) extract exhibited the strongest antioxidant activity in a DPPH radical-scavenging assay and this was compared with the positive control, α-tocopherol. This extract was further divided into eight fractions, designated as F1–F8, by silica gel liquid chromatography. In most cases, F4 and F8 were found to possess the strongest antioxidative activities. Significant associations between the antioxidant potency and the total phenolic content, as well as between the antioxidant potency and free amino groups, were found for the supernatant, extract, and fractions. With this method, squid pen waste can be utilized and has potential in the production of functional foods.

1. Introduction

Squid pen waste is an important source of bioactive molecules. The major components (on dry weight basis) of squid pen waste are protein (61%), chitin (38%), and minerals (1%) (Wang et al., 2006). Bioconversion of chitinous materials has been proposed as a waste treatment alternative to the disposal of shellfish wastes (Synowiecki & Al-Khateeb, 2000; Wang, Wang, & Huang, 2008; Wang & Yeh, 2006). However, so far, there are few reports about the bioactive compounds from the fermentation of squid pen waste. As squid pen waste undergoes rapid putrefaction, it is necessary to preserve the material by adopting environmentally safe techniques, prior to production of bioactive components for biotechnological and pharmaceutical applications.

The fermentation technique can be used for utilization of squid pen waste (Wang, Lin, Liang, Liu, & Kuo, 2009; Wang et al., 2006). During fermentation, due to liquefaction of protein and chitin, bioactive material-rich liquor is formed, including peptides, amino acids and chitooligosaccharides (Wang et al., 2009). Recently, we have evaluated and reported the DPPH free radical-scavenging activities of the squid pen powder (SPP) fermented supernatants (Wang et al., 2009). These results have encouraged us to pay more attention to the fermentation of squid pen. Literature survey revealed that shellfish waste is a rich source of phenolic compounds (Seymour, Li, & Morrissey, 1996). Phenolic compounds play an important role in the antioxidative properties and phenolic substances were also reported to possess a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory and anticancer activity (Fernandez-Panchon, Villano, Troncoso, & Garcia-Parrilla, 2008; Singh, Arseneault, Sanderson, Murthy, & Ramassamy, 2008). Besides, the SPP-fermented supernatant may also be rich in certain compounds with amino groups, to enhance its antioxidant properties. It is expected that this bioactive material-rich liquor will have beneficial biological functions due to the inherent protein and chitin hydrolysis and bioactive material production occurring during fermentation. Protein hydrolysates are known to possess strong antioxidative properties (Kim & Mendis, 2006).

In this study the antioxidant properties of the liquor obtained during fermentation of squid pen waste was assessed, using different *in vitro* systems. We further investigated and evaluated the antioxidant activities of the solvent extract and fractions of the fermented supernatant compared to synthetic antioxidants, such as α-tocopherol. In addition, we also assessed the phenolic content, free amino groups and the DPPH free radical-scavenging activity of the fermented supernatant, extract and fractions. Furthermore,

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the correlation between the antioxidant activity and phenolic content, as well as between the antioxidant activity and free amino groups, was also considered.

2. Materials and methods

2.1. Materials

The squid pen powder (SPP), shrimp shell powder (SSP), and crab shell powder (CSP) used in these experiments were prepared as described earlier (Wang, Shin, Liang, & Wang, 2002). Squid pens, shrimp shells, and crab shells were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). The squid pens, shrimp shells, and crab shells were washed thoroughly with tap water and then dried at 35 °C. The dried materials obtained were milled to powder for use as the carbon source for antioxidant materials production. *Serratia ureilytica* TKU013, for the experiment, was obtained previously from soils and maintained on nutrient agar. α,α-Diphenyl-β-picrylhydrazyl (DPPH·) and Folin–Ciocalteu reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and solvents used were of the highest grade available.

2.2. Biowaste fermentation for preparation of antioxidant materials

S. ureilytica TKU013 was isolated from the soil in Taiwan and maintained on nutrient agar plates. In the investigation of the culture condition, growth was carried out in a basal medium containing 0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7), and supplemented with 0.5–2% (w/v) of various carbon sources for 1–4 days of fermentation. The carbon sources investigated included SPP, SSP, CSP, chitin, chitosan, and *Paecilomyces cicadae*. The fermented broths were centrifuged (4 °C and 8200*g* for 20 min) and the supernatants were collected for measurement of antioxidant activity, using the procedure described below.

2.3. Measurement of DPPH radical-scavenging activity

The diluted culture supernatant (150 μl) was mixed with 37.5 μl of methanolic solution containing 0.75 mM DPPH radical. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The scavenging ability was calculated as follows: Scavenging activity (%) = $[(A_{517}$ of control $- A_{517}$ of sample$)/A_{517}$ of control$] \times 100$.

2.4. Measurement of Fe^{2+}-chelating ability

The method of Decker and Welch (1990) was adopted. Five millilitres of the diluted culture supernatants were spiked with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine solutions. After reaction for 10 min, the absorbance (at 562 nm) of the resulting solution was recorded. A complex of Fe^{2+}/ferrozine has a strong absorbance at 562 nm. A high ferrous ion-chelating ability in the test sample results in a low absorbance. The ferrous ion-chelating ability was calculated as follows: Chelating ability (%) = [1 − (test sample absorbance/blank sample absorbance)] × 100.

2.5. Measurement of reducing power

A method developed by Oyaizu (1986) for testing reducing power was used. The diluted culture supernatant or distilled water (control) (0.5 ml) was spiked with 0.5 ml of sodium phosphate buffer (0.02 M, pH 7) and 0.5 ml of 1% potassium ferricyanide. The mixture was then kept in a 50 °C water bath for 20 min. The resulting solution was cooled rapidly, spiked with 0.5 ml of 10% trichloroacetic acid, and centrifuged at 800*g* for 10 min. The supernatant (1.5 ml) was then mixed with 0.2 ml of 0.1% ferrichloride. After reaction for 10 min, the absorbance at 700 nm was measured. A high absorbance was indicative of strong reducing power.

2.6. Determination of total phenolic content

The total phenolics of the culture supernatants were determined (Julkunen-Tiitto, 1985) and expressed as microgrammes of gallic acid equivalents per millilitre of culture supernatant. Two millilitres of deionized water and 1 ml of Folin–Ciocalteu's phenol reagent were added to 0.3 ml of each sample. Five millilitres of 20% aqueous sodium carbonate solution (w/v) were added and mixed well, and then the mixture was allowed to stand at ambient temperature for 20 min. Absorbance of the developed dark bluepurple colour was measured by spectrophotometer at 735 nm. The content of total phenolics in each sample was determined using a standard curve prepared with gallic acid at varied concentrations (0, 50, 100, 200, 400, 600, and 800 μg/ml).

2.7. Extraction and isolation of fermented supernatants

For the production of antioxidants, *S. ureilytica* TKU013 was grown in 50 ml of liquid medium in an Erlenmeyer flask (250 ml) containing 1.5% SPP, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7). One millilitres of the seed culture was transferred into 50 ml of the same medium and grown in an orbital shaking incubator for 3 days at 25 °C and pH 7 (the pH after being autoclaved was 7.5). After incubation, the culture broth was centrifuged (4 °C and 12,000*g* for 20 min), and the supernatant was used for further extraction and isolation.

The culture supernatant (4.5 l) was extracted with the equivalent volume of ethyl acetate (EA) for 12 h, three times at room temperature. Since the EA layers exhibited the highest activity in the subsequent antioxidant assays, they were combined, collected and concentrated under reduced pressure to dryness, yielding the crude extract, which was then suspended in CH_2Cl_2. The solution was separated by MPLC silica gel column chromatography, eluted with CH_2Cl_2/MeOH from 100:0 to 0:100. Eight fractions (F1–F8) were obtained, and then solvent in each fraction was evaporated to dryness under vacuum. All of the eight fractions were assayed for DPPH radical-scavenging activity. Fraction 4 (F4) and fraction 8 (F8) showed higher activities, but the amount of F4 was 2–3 times higher than that of F8; F4 was further purified by repeated column chromatography on Sephadex LH-20 (MeOH) to obtain a pure compound. The scheme of separation and purification is shown in Fig. 1.

2.8. Thin-layer chromatography analysis

The active EA extract (0.317 g) was subjected to the column chromatography on silica gel and eluted with a solvent mixture of CH_2Cl_2/MeOH (100:0–0:100, v/v), affording eight major fractions. TLC analysis was performed on silica gel using CH_2Cl_2/MeOH in various proportions as the mobile phase. Silica Gel TLC plates (0.25 mm) were obtained from Merck. Compounds were detected by spraying *p*-anisaldehyde–ethanol–sulphuric acid mixture, followed by heating at 100 °C for 5 min. Another system was analysed by using 5:4:3 (v/v/v) *n*-butanol/methanol/16% aqueous ammonia as the mobile phase (Kadokura et al., 2007). After developing the TLC plates, the compounds were visualized by spraying with an aqueous solution of 2.4% (w/v) phosphomolybdic acid, 5% (v/v) H_2SO_4, and 1.5% (v/v) H_3PO_4 (phosphomolybdic acid reagent) or ethanol containing 0.5% (w/v) ninhydrin (ninhydrin reagent) or the DPPH· solution described above, followed by heating.

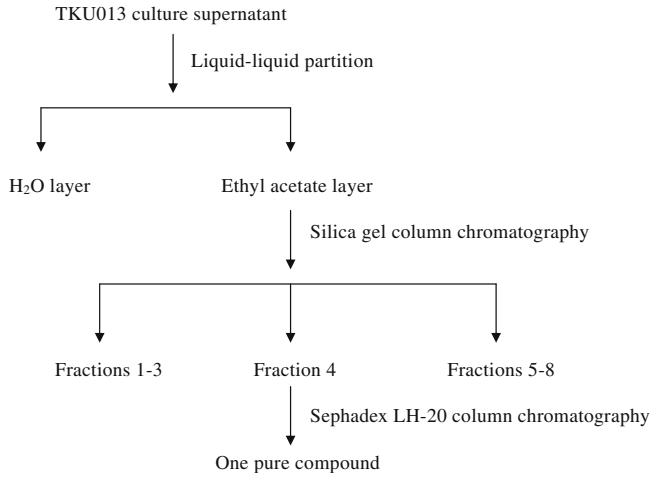

Fig. 1. Scheme of the extraction and separation of antioxidative materials from fermented squid pen biowaste by *S. ureilytica* TKU013.

2.9. Statistical analysis

In this study, each experiment was conducted in triplicate. All statistics analyses were carried out using SPSS 11.01 for Windows. To determine whether there were any differences between activities of samples, variance analysis was applied to the result. Values of $p < 0.05$ were considered as significantly different ($\alpha = 0.05$).

3. Results and discussion

3.1. Culture conditions and antioxidant production

To utilize the chitin/protein-containing biowastes, we incubated *S. ureilytica* TKU013 for 1–5 days at 25 °C, by using SPP, SSP, CSP, chitin, chitosan, or *P. cicadae* (0.5–2%) as carbon/nitrogen sources, and analysed the antioxidant activity of the culture supernatant using DPPH·-scavenging ability. As shown in Table 1, TKU013 culture supernatant (1.5% SPP) incubated for 4 days had the highest antioxidant activity; the DPPH·-scavenging ability of TKU013 culture supernatant was about 82%. To analyse the antioxidant activity of the culture medium at the 0 day, we heated these biowastes in an autoclave (121 °C for 15 min), the antioxidant activities (26–35%) were found in the supernatants (shrimp shell powder, crab shell powder, squid pen powder); 50% antioxidant activity was found in *P. cicadae* powder supernatant, but no antioxidant activity was found in chitin powder or chitosan powder supernatant. However, as shown in Table 1, it was found that the antioxidant activities increased after fermenting by TKU013. Squid pen powder was the most suitable carbon/nitrogen source for antioxidant production by strain TKU013 (Table 1). It is assumed that, even though the treatment (121 °C for 15 min) degrades these biowastes and produces some of the antioxidant materials, most of the antioxidant materials are produced by strain TKU013.

Table 1
Summary of the optimal culture conditions for antioxidant production by *S. ureilytica* TKU013 grown on the six preparations.

Carbon/nitrogen source	Concentration (%)	Day of maximum antioxidant activity	Maximum antioxidant activity (%)
SSP	1.5	1	60 ± 3a
CSP	2	1	50 ± 2b
SPP	1.5	4	82 ± 3c
Chitin	2	1	38 ± 2d
Chitosan	1.5	1	4 ± 1e
P. cicadae	1.5	1	60 ± 5a

All data are expressed as means ± SD from three different experiments (each experiment was conducted in triplicate). Data with different letters are significantly different at $p < 0.05$.

In addition, as shown in Table 1, carbon/nitrogen sources with protein and chitin were more suitable as inducers for antioxidant production by *S. ureilytica* TKU013 than were the carbon/nitrogen sources with only chitin but no protein. The production of the antioxidant might be related to the protein in the carbon/nitrogen sources. Deproteinization of protein-containing materials (SPP, SSP, CSP, and *P. cicadae* powder) by alkali (Wang et al., 2006) was also used to obtain carbon/nitrogen sources, and the relationships between incubation time and antioxidant activity were investigated. As shown in Fig. 2(A), maximum antioxidant activity was still found at the 4th day, with 1.5% SPP as carbon/nitrogen source.

DPPH· possesses a hydrogen free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers. Further, it is well accepted that the DPPH free radical-scavenging by antioxidants is due to their hydrogen-donating ability. As reported previously (Wang et al., 2009), it was demonstrated that antioxidant oligopeptides and chitooligosaccharides were present in the culture supernatant. The increase in oligopeptides and chitooligosaccharides resulted in more active amino groups and these could donate more hydrogen to react with DPPH radical. Therefore, SPP-fermented supernatant at the 4th day, with the highest protein concentration and reducing sugar (Wang et al., 2009) had the strongest scavenging effect on the DPPH radical.

3.2. Chelating effects on ferrous ion

Ferrous ion-chelating abilities of the supernatants, fermented from 1.5% SPP, SSP, CSP, SPP-NaOH, SSP-NaOH, or CSP-NaOH for 1–4 days at 25 °C, were analysed. As shown in Fig. 2(B), the culture supernatants (SPP and SSP) incubated for 2–4 days had the highest chelating ability (82–90%) on ferrous ion. The chelating ability did not decrease with the culture time. Therefore, the antioxidants in SPP- and SSP-fermented supernatants are stable for the culture time. The chelating activity of CSP-fermented supernatant was the lowest for 4 days. However, CSP-fermented supernatant at the 1st day showed 50% DPPH·-scavenging ability (Fig. 2(A)) and this was higher than those of SPP-NaOH, SSP-NaOH, or CSP-NaOH. Since ferrous ions are the most effective pro-oxidants, the higher chelating abilities of SPP- and SSP-fermented supernatants would be beneficial. For CSP-fermented supernatant, the difference between DPPH·-scavenging ability and ferrous ions chelating ability might be related to the antioxidant compounds. Previous studies showed that the chelating to ferrous ion was dependent on the numbers of hydroxyl, and that the hydroxyl substitution in the *ortho* position was desirable (Wang, Gao, Zhou, Cai, & Yao, 2008). The low chelating effect of CSP-fermented supernatant was probably due to the chemical structures of the antioxidant compounds, which were not suitable for combining with the metal ion. Therefore, the antioxidant compounds are different in various carbon/nitrogen sources.

3.3. Reducing power

The reducing powers of various culture supernatants from SPP, SSP, CSP, SPP-NaOH, SSP-NaOH, or CSP-NaOH are shown in Fig. 2(C). It was found that, at 3% (v/v), the culture supernatants (1.5% SPP) incubated for 2–4 days have the highest reducing power (0.63–0.66). The reducing power did not decrease with the culture time. Therefore, the antioxidants in SPP-fermented supernatant are stable for the culture time. At 3% (v/v), SSP-fermented supernatants (for 1–4 days) showed moderate reducing power (0.18–0.27). At a higher concentration, e.g., 30% (v/v), CSP-fermented supernatants

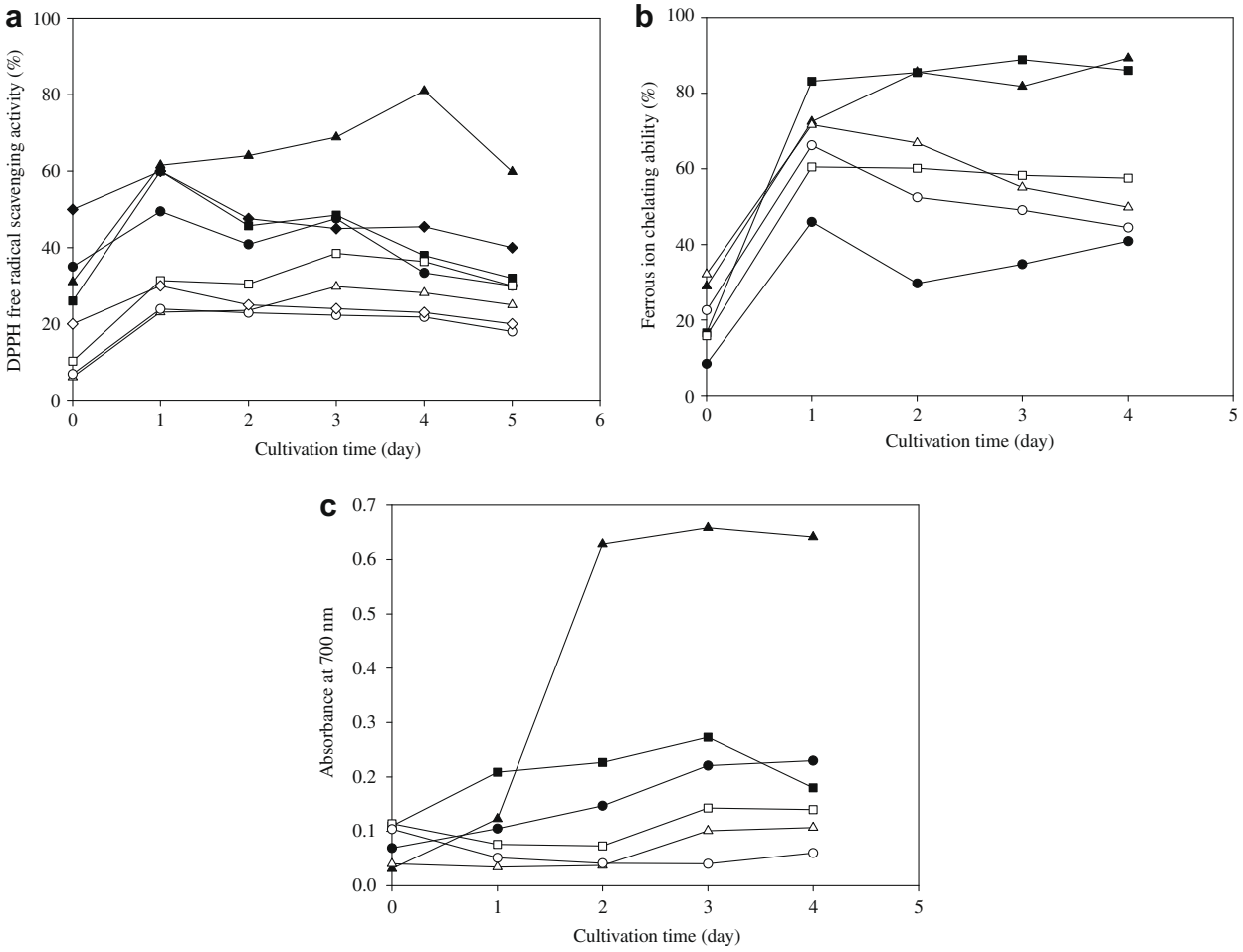

Fig. 2. Antioxidant activities of TKU013 culture supernatants in various media. (A) DPPH free radical-scavenging ability; (B) ferrous ion-chelating ability; (C) reducing power. (▲) SPP; (■) SSP; (●) CSP; (♦) *P. cicadae*; (△) SPP-NaOH; (□) SSP-NaOH; (○) CSP-NaOH; (◇) *P. cicadae*-NaOH. The concentrations of SPP- and SSP-fermented supernatants were 3% (v/v); other samples were 30% (v/v) in reducing power analysis.

(for 1–4 days) showed slight reducing power (0.11–0.23). The reducing power could be attributed mainly to the bioactive compounds associated with antioxidant activity (He, Chen, Sun, Zhang, & Gao, 2006; Lin & Chou, 2004; Seymour et al., 1996; Xing et al., 2005). These bioactive compounds might be present in SPP-fermented supernatant, including phenolics, oligopeptides, or chitooligosaccharides; they are good electron donors and can terminate the radical chain reactions by converting free radicals to more stable products.

3.4. Total phenolic content

It has been reported that shrimp shell waste contains natural antioxidants, mainly phenolic compounds (Seymour et al., 1996). The contents of total phenolics (TPs) in various fermented supernatants from SPP, SSP, CSP, SPP-NaOH, SSP-NaOH, or CSP-NaOH are shown in Fig. 3. It was found that the fermented supernatant (1.5% SPP) incubated at the 3rd and 4th day had the highest TPs contents, with 538–549 μg of gallic acid equivalents (GAE) per millilitre of the fermented supernatant. The SSP-fermented supernatants (for 1–4 days) contained little TPs (122–142 μg/ml GAE) as compared to SPP-fermented supernatants. However, no TPs content was determined in CSP and CSP-NaOH fermented supernatants. SPP-fermented supernatant, at the 4th day, with high phenolic content, also showed high DPPH radical-scavenging activity (Table 1). This result suggested that phenolic compounds might be responsible for the activity.

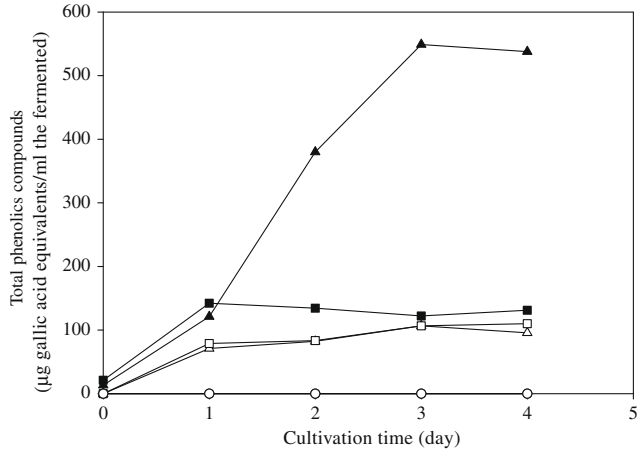

Fig. 3. Total phenolic contents of TKU013 culture supernatants in various media. (▲) SPP; (■) SSP; (●) CSP; (♦) *P. cicadae*; (△) SPP-NaOH; (□) SSP-NaOH; (○) CSP-NaOH; (◇) *P. cicadae*-NaOH.

3.5. Antioxidant activity of SPP-fermented supernatant extract and fractions

The DPPH radical-scavenging assay method has been developed and applied in recent years to screen and evaluate the total antioxidant activity of the extracts of biological preparations (Huang

Table 2
The values of percent DPPH·-scavenging activities and EC_{50} of SPP- and TKU013-fermented supernatant extracts.[a]

Sample	DPPH·-scavenging activity[b] (%)	EC_{50}[c] (μg/ml)
EA extract of SPP	27.6 ± 0.9a	136 ± 3.2a
Aqueous residue of SPP	8.3 ± 0.3b	148 ± 6.4b
EA extract of supernatant	99.1 ± 1.2c	8.5 ± 0.3c
Aqueous residue of supernatant	32.1 ± 0.8d	48.2 ± 2.2d
α-Tocopherol	–	12.2 ± 0.5e

[a] All data are expressed as means ± SD from three different experiments (each experiment was conducted in triplicate). Data in the same column with different letters are significantly different at $p < 0.05$.
[b] The concentration of all samples was 200 μg/ml.
[c] EC_{50} value: DPPH radical was scavenged by 50%. EC_{50} value was obtained by interpolation from linear regression analysis.

et al., 2008; Zhang et al., 2008). In the present work, the DPPH radical-scavenging assay was successfully used for the evaluation of the antioxidant activity of the crude extract and fractions derived from SPP-fermented supernatant. The assay method has also been successfully used in our previous reports (Wang, Peng, Liang, & Liu, 2008; Wang et al., 2009).

The DPPH·-scavenging activities of SPP- and TKU013-fermented supernatant extracts were summarised in Table 2. As shown in Table 2, the highest percent DPPH radical-scavenging activity was observed in the EA extract of TKU013-fermented supernatant, while the other samples, including the EA extract and aqueous residue of SPP, and aqueous residue of TKU013-fermented supernatant, showed lower scavenging activities. This result suggested that compounds with the strongest radical-scavenging activity in TKU013-fermented supernatant are of medium polarity. At all concentrations tested, the EA extract of TKU013-fermented supernatant exhibited a dose-dependent DPPH radical-scavenging activity (data not shown). As shown in Table 2, the EA extract of TKU013-fermented supernatant gave the lowest EC_{50} value of 8.56 μg/ml in the DPPH· assay. The EA extract of TKU013-fermented supernatant had a better activity than had the positive control, α-tocopherol (EC_{50} = 12.15 μg/ml). These results demonstrated that the EA extract of TKU013-fermented supernatant is a fairly good scavenger for DPPH radicals.

Fig. 4 shows the DPPH radical-scavenging activities of fractions isolated from the EA extract of TKU013 SPP-fermented supernatant by liquid chromatography. As indicated, all of the eight fractions (F1–F8) showed a concentration-dependent DPPH·-scavenging activity over the eight tested concentration levels from 1.56 to 200 μg/ml. In addition, F4 and F8 showed higher activity than did the EA extract itself.

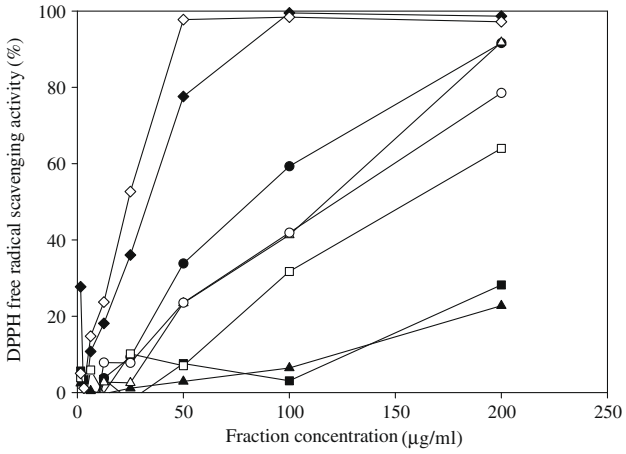

Fig. 4. DPPH free radical-scavenging activities of the different fractions from the EA extract of TKU013 SPP-fermented supernatant after silica gel column chromatography. (▲) F1; (■) F2; (●) F3; (♦) F4; (△) F5; (□) F6; (○) F7; (◇) F8.

3.6. Determination of the antioxidative components

In an attempt to elucidate the antioxidative components in TKU013-fermented supernatant, the contents of total polyphenols in F1–F8 were then determined. F8 had the highest concentration of total polyphenols (2.85 ± 0.06 mg of gallic acid equivalents/g of dry weight), followed by F4 (2.62 ± 0.03 mg of gallic acid equivalents/g of dry weight). Data analysis revealed that fractions with high phenolic contents (data not shown) also showed high DPPH radical-scavenging activity (Fig. 4). Because the amount of F4 was 2–3 times higher than that of F8, F4 was selected for further study. The antioxidant compounds in F4 were analysed using TLC. The fraction was developed on the TLC plates, and compounds only visualized by spraying with anisaldehyde reagent, ninhydrin reagent, phosphomolybdic acid reagent, and DPPH· solution (data not shown). Furthermore, certain nitrogen compounds of TKU013 culture supernatant were detected by the method of Ikawa, Schaper, Dollard, and Sasner (2003). TKU013 culture supernatant reacted strongly with Folin–Ciocalteu phenol reagent (shown by colorimetric means and TLC) (Fig. 5). Therefore, the antioxidative

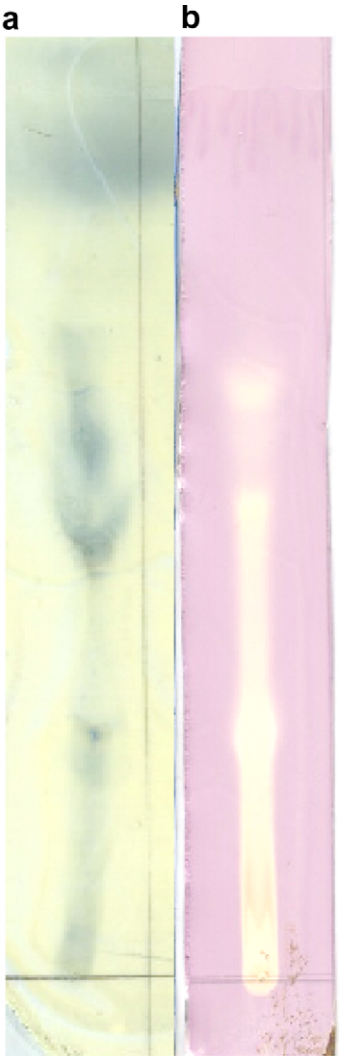

Fig. 5. TLC analysis of the antioxidative compounds produced by *S. ureilytica* TKU013 using SPP as carbon/nitrogen source. After developing the TLC plates, the antioxidants with certain nitrogen compounds were visualized using the following reagents: a, Folin–Ciocalteu phenol reagent; b, DPPH· solution.

components in TKU013 culture supernatant might contain nitrogen compounds. These results of TLC analysis suggest that the antioxidant compound in F4 might have free amino groups and phenolic compounds. However, it was difficult to use the current data to elucidate the chemical structure of the compound in the fraction unless it was compared with the respective authentic standards. We will further confirm the structure by NMR, IR and mass spectral analysis in the future.

4. Conclusion

In summary, antioxidative compounds were obtained from the culture supernatant of *S. ureilytica* TKU013, using squid pen as the sole carbon/nitrogen source. In addition, it was also found that the culture supernatant had antioxidant activity as well. The results in the present study clearly demonstrate that the EA extract of TKU013-fermented supernatant is a fairly active fraction for *in vitro* DPPH free radical-scavenging activity. The antioxidative characteristics of TKU013-fermented supernatant, extract, and fractions were determined in multiple ways, including the measurement of DPPH free radical-scavenging activity, Fe^{2+}-chelating ability, reducing power and phenolic content. In addition, the results suggest that free amino groups and phenolic compounds might be major contributors to the antioxidative activities of TKU013-fermented supernatant. Further efforts are underway to isolate and identify the active compounds from this fermented supernatant. However, considering that the 4th day fermented supernatant is highest in phenolics and the 2nd day fermented supernatant is highest in reductive activity, some factors other than free amino groups and phenolic compounds appear to also be responsible for the observed effects.

Our data provide a useful example of utilizing squid pen biowaste material as a valuable functional ingredient. The findings of the current report appear useful for further research aiming to isolate and identify the specific compounds responsible for the antioxidant activity of TKU013-fermented supernatant.

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