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Application of fucoidan for the encapsulation of yeast K2 toxin

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ABSTRACT

Fucoidan is an anionic polysaccharide derived from brown algae and is composed of L-fucose residues linked by alternating α-(1→3) with α-(1→4) linkages or by either α-(1→3) or α-(1→4) linkages alone. Fucoidan is known for its pharmacological, antibacterial, and antifungal properties and is used in the food industry. The aim of the study was to apply fucoidan to the formulation of the yeast killer toxin K2 produced by *Saccharomyces cerevisiae*. K2 toxin-loaded fucoidan particles were formed by a low-cost complexation method. They were characterised by dynamic light scattering, FT-IR spectroscopy, and scanning electron microscopy methods. The particles ranged in size from 180 to 280 nm and were stable for at least one month at 4°C. The encapsulated K2 toxin retained its antimicrobial activity. In addition, complexation with fucoidan increased the stability of the K2 toxin at room temperature. Fucoidan could serve as a carrier for K2 toxin and enhance the biotechnological potential of antimicrobial agents produced by *S. cerevisiae*. Encapsulation of K2 toxin opens up opportunities for its use in the food and beverage industry for biopreservation.

1. Introduction

Nowadays, macroalgae-derived polysaccharides are attracting more and more attention. Firstly, there are significant biomass resources for their production. Currently, about thirty-two million tons of macroalgae are harvested worldwide, and polysaccharides constitute from 5 % to 75 % of their dry weight. Secondly, macroalgae-derived polysaccharides are of interest because of their potential industrial applications and biological activities. Many of them are sulphated and not found in terrestrial plants (Otero et al., 2023).

Fucoidan is found in brown seaweed. Its structure is heterogeneous and varies depending on the species of brown algae, their cultivation conditions, harvest time, and the method of fucoidan extraction. A chemical backbone is formed from l-fucose residues linked by alternating α-(1→3) with α-(1→4) linkages or by either α-(1→3) or α-(1→4) linkages alone. In the backbone branching, α-(1→2) linkages are sometimes present. Other monomers such as galactose, glucose, xylose, and galacturonic acid have also been identified in the structure of fucoidans. The C-2 and/or C-4 positions are substituted by sulphate ester groups. Depending on the glycosidic linkage, a sulphate group can also be attached to C-3 (Li, Lu, Wei & Zhao, 2008; Zayed & Ulber, 2019; Zayed, Cao, Trang, & Ulber, 2023b; Zayed, El-Aasr, Ibrahim & Ulber, 2020).

Fucoidan is known for its pharmacological properties and exhibits immunomodulatory, antiviral, anticoagulant, anti-inflammatory, anti-hyperglycemic, antitumor, and anti-angiogenic bioactivities (Shi et al., 2024; Wu et al., 2024; Yu et al., 2024; Zayed & Ulber, 2020). Furthermore, fucoidan has also found its application in the food industry (Mensah, Kanwugu, Panda & Adadi; 2023). In the EU, fucoidan extracts from *Fucus vesiculosus* and *Undaria pinnatifida* have been approved for use in food supplements and have been recognised as a novel food. An intake of up to 250 mg of fucoidan per day is permitted for oral consumption. These extracts have also been approved as GRAS in the United States of America (Jayawardena et al., 2022; Lahteenmaki-Uutela et al., 2021).

Fucoidan is also known for its antibacterial and antifungal properties. Fucoidan has been shown to be more effective against Gram-positive bacteria than Gram-negative. The mechanism of inhibition is based on its binding to membrane proteins and disruption of the bacterial cell membrane. Antibacterial properties depend on the sulphate

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group content in the fucoidan structure (Mensah, Kanwugu, Panda & Adadi; 2023; Ayrapetyan et al., 2021). Recently, a fucoidan-rich extract of *Fucus vesiculosus* was found to inhibit food pathogens such as *Listeria monocytogenes* at a concentration of 0.2 mg/mL (Graikini, Soro, Sivagnanam, Tiwari & Sanchez, 2023). Several studies have demonstrated antifungal properties of fucoidan extracts. Phull et al. (2017) found that *Aspergillus flavus, Aspergillus fumigatus* and *Mucor* spp. were inhibited by the fucoidan extract of *U. pinnatifida*. Fucoidan also had a fungicidal effect on *Candida* species, and the minimum fungicidal concentration was 0.1 μg/mL of fucoidan (Mensah, Kanwugu, Panda & Adadi; 2023).

Fucoidan has been used for the formulation and delivery of nutraceuticals (Zhang, Wei & Xue, 2022) and chemotherapeutics (Hwang, Lin, Kuo & Hsu, 2017). For this purpose, it can also be combined with other biopolymers. Recently, a zein-fucoidan composite has been developed for the delivery of resveratrol and pterostilbene (Liu, Chen, Qin, Jiang & Zhang, 2020; Liu, Qin, Jiang, Chen, & Zhang, 2022). For the delivery of carotenoids, fucoidan has been used for nanoemulsion stabilisation as a natural emulsifier (Oliyaei, Moosavi-Nasab & Tanideh, 2022) or as a biopolymer to develop a triple layer complex in combination with xylan and chitooligosaccharides (Straksys, Gruskiene, Matulaitiene, Kavleiskaja & Melo, 2023).

In this study, we hypothesise that fucoidan could be used to formulate the yeast killer K2 toxin produced by *Saccharomyces cerevisiae.* It is an extrachromosomally encoded protein of 21.5 kDa (Dignard, Whiteway, Germain, Tessier, & Thomas, 1991). Usually, killer toxins are active against non-killer strains and strains that produce other types of killer toxins. A toxin producer is immune to its toxin (Schaffrath, Meinhardt, & Klassen, 2018). The mode of action of not all types of killer toxin is fully understood. To kill cells, the K2 toxin causes membrane permeabilization. Initially, K2 binds to β-1,6-glucan on the cell wall and then interacts with Kre1p as a plasma membrane receptor causing the formation of ion channels (Novotna, Flegelova & Janderova, 2004; Lukša et al., 2015; Orentaite, Poranen, Oksanen, Daugelavicius & Bamford, 2016; Schaffrath, Meinhardt & Klassen, 2018; Mannazzu et al., 2019). In general, the killer phenotype is widespread among yeast, and the targets of killer toxins are also spoilage and pathogenic microorganisms. However, the biotechnological potential of yeast killer toxins as natural antimicrobials has not yet been fully exploited. In the food industry, they could serve as biocontrol agents and reduce the use of chemical ones (Mannazzu et al., 2019). The different stability of killer toxins as proteins under different environmental conditions is one of the limiting factors. Here, for the first time, the killer toxin K2 was encapsulated using fucoidan. The K2 toxin complexed with the polysaccharide retained its killing activity and showed an increase in stability.

2. Experimental

2.1. Materials

Fucoidan from *F. vesiculosus* (98 %) was purchased from Sigma-Aldrich. The content of sulfur was 8.89 % and its molecular mass was equal to 95 kDa (Hahn et al., 2016). SP-Sepharose fast flow was purchased from GE Healthcare, CM-Sepharose was obtained from Pharmacia Fine Chemicals. Bicinchoninic acid was obtained from Sigma-Aldrich.

2.2. Yeast strains and media

S. cerevisiae strain M437 (*wt, HM/HM [Kil-K2]*) was used for the isolation of killer toxin K2 (Gulbiniene et al., 2004). For the analysis of the killing activity and stability of the purified killer protein K2, *S. cerevisiae* strain α'1 (*MAT α leu2-2 [Kil-0]*) was applied (Gulbiniene et al., 2004). For the analysis of encapsulated toxin activity, yeast strains *Candida albicans, Pichia anomala, P. holsti, P. membranifaciens, S. cerevisiae,* and *Torulaspora delbrueckii* yeast strains (the collection of the Laboratory of Genetics, Nature Research Centre, Lithuania) were used.

YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) was used for yeast amplification. MBA medium (5 g/L yeast extract, 5 g/L peptone, 20 g/L dextrose, 10.5 g/L citric acid, 35.3 g/L $Na_2HPO_4 \times 12H_2O$, 20 g/L agar, 20 mg/L methylene blue dye) adjusted to pH 4.0 was used for detection of killing activity. SC medium (20 g/L dextrose, 2 g/L K_2HPO_4, 1 g/L $MgSO_4 \times 7H_2O$, 1 g/L $(NH_4)_2SO_4$, 12.9 g/L citric acid, 27.6 g/L $Na_2HPO_4 \times 12H_2O$) adjusted to pH 4.0 and containing 50 g/L glycerol was used for the cultivation of K2 toxin producing strain.

2.3. Purification of yeast K2 toxin

K2 toxin-producing *S. cerevisiae* strain M437 (*wt, HM/HM [Kil-K2*]) was grown in liquid synthetic medium SC (20 g/L dextrose, 2 g/L K_2HPO_4, 1 g/L $MgSO_4 \times 7H_2O$, 1 g/L $(NH_4)_2SO_4$, 12.9 g/L citric acid, 27.6 g/L $Na_2HPO_4 \times 12H_2O$, 50 g/L glycerol, pH 4.0) for 4 days at 18 °C with gentle shaking (40 rpm) until a cell density (OD_{600}) of approximately 0.6–0.8 was reached. The yeast cells were removed by centrifugation at 5000 × *g* for 10 min and the supernatant was filtered through a 0.22 μm sterile polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA) and used to measure the killing activity. The supernatant containing K2 toxin activity was then concentrated 40-fold using the Amicon pressure-based system (MWCO 10 kDa membrane) (Sigma-Aldrich, St. Louis, MO, USA) and diluted with deionised water to achieve a conductivity of 2.0 mS/cm. The solution was then loaded onto a SP-Sepharose column (1.5 × 8 cm) previously equilibrated with 50 mM sodium acetate buffer pH 4.2 at a flow rate of 1.5 mL/min. Unbound proteins were washed out with the same buffer. The bound proteins were eluted with ten-bed volumes of a linear NaCl gradient (0–0.3 M) in 50 mM sodium acetate buffer pH 4.2. The fractions containing killing activity were pooled and dialysed against 50 mM sodium acetate buffer pH 4.6 using a dialysis tube (MWCO 3.5 kDa). A dialysed solution was reloaded on the same column equilibrated with 50 mM sodium acetate buffer pH 4.6. The unbound protein fractions were pooled, dialysed against 50 mM sodium acetate pH 4.2, and loaded onto a CM-Sepharose (1 × 6 cm) column previously equilibrated with the same buffer at a flow rate of 0.5 mL/min. The K2 toxin bound to CM-Sepharose was eluted with ten-bed volumes of a linear NaCl gradient (0–0.3 M). The active fractions were collected and sodium chloride was removed by dialysis against 50 mM sodium acetate buffer pH 4.2. The active protein solution was then used for complexation with fucoidan. The protein concentration was determined by the bicinchoninic acid method. Bovine serum albumin was used to construct a calibration curve. The samples were assayed using the microprocedure with a sensitivity range of 1–25 μg protein/mL (Simpson, 2003). From 2 L of yeast culture medium, 67±34 μg of toxin was obtained.

2.4. Fucoidan complexation with yeast K2 toxin

The K2 toxin solution was prepared in 50 mM sodium acetate buffer pH 4.2 at a concentration of 4.3 ± 0.6 μg/mL. The fucoidan solution was prepared in deionised water at a concentration of 5.0 mg/mL and the pH was adjusted to the value of 3.5, 4.2, or 4.8 using 0.1 M NaOH or 0.1 M HCl. The solution was then filtered through cellulose acetate filters with a pore size of 0.2 μm. To form particles, a desired amount of K2 solution was added dropwise to the fucoidan solution under constant stirring at room temperature and the pH value of the mixture was further adjusted to 3.5, 4.2 or 4.8. Before the addition of the K2 solution, if necessary, an appropriate amount of water was added to obtain a final fucoidan concentration of 0.1 or 0.4 mg/mL and a final K2 concentration of 2.6 or 4.0 μg/mL. Therefore, the fucoidan and K2 concentrations were 0.1 mg/mL and 2.6 μg/mL (the particles F_1-$K2_a$), 0.1 mg/mL and 4.0 μg/mL (the particles F_1-$K2_b$), 0.4 mg/mL and 2.6 μg/mL (the particles F_2-$K2_a$) and 0.4 mg/mL and 4.0 μg/mL (the particles F_2-$K2_b$), respectively. The samples were then gently shaken at 150 rpm for 24 h at 4 °C. The particles obtained were stored at 4 °C for further analysis.

2.5. Physicochemical characteristics of K2 toxin-loaded fucoidan particles

For the determination of particle diameter, a Zetasizer Nano ZS instrument (Malvern Instruments) equipped with a 4 mV HeNe laser at a wavelength of 633 nm was used. The intensity of the scattered light was recorded at an angle of 173°. All measurements were performed at 20 °C. Data were analysed using the Malvern Zetasizer software 7.03. The same instrument was used to determine the zeta potential of the particles.

Fourier-transform infrared (FT-IR) spectra were recorded on a Perkin-Elmer Frontier 65 spectrometer using the Universal ATR Sampling Accessory. All spectra were an average of 100 scans from 4000 to 600 cm^{-1} with a resolution of 2 cm^{-1}. The solutions of fucoidan and K2 toxin-loaded fucoidan complex were freeze-dried. Before freeze-drying, sodium acetate was removed using ultrafiltration tubes (Ultracel®, cut-off 10 kDa, Merck Millipore Ltd), concentrating the solution and re-diluting several times with acidified deionised water (approximately pH 4.5).

Raman spectra were recorded using a MultiRAM FT-Raman spectrometer (Bruker Optik GmbH, Germany) equipped with a 1064 nm, 1000 mW Nd:YAG laser. The samples were analysed in powder form. Spectra were collected over the range 3500–800 nm. The measurement conditions for pure fucoidan were as follows: a laser power of 250 mW and an exposure time of 60 s. For the samples of K2 toxin-loaded particles, the lower laser power of 40 mW and an exposure time of 15 s were used.

UV–vis absorption spectra were recorded on an Ultrospec 4000 spectrophotometer (Pharmacia Biotech, Sweden) using a 1 cm quartz cell.

For scanning electron microscopy (SEM), a Hitachi SU-70 (FE-SEM, Hitachi, Tokyo, Japan) scanning electron microscope was used. The SEM images were examined at an acceleration voltage of 2.0 kV. Samples were diluted five times with acidified deionised water and dropped onto a glass slide sputter-coated with a 10-nm silver layer. Then, they were gently dried at room temperature and again sputter-coated with 10-nm silver.

2.6. Measurement of killing activity

2.6.1. Inhibition zone assay

To determine K2 toxin activity during the purification process, 20 µl of crude extract or purified killer protein sample were spotted onto MBA plates, pH 4.0, inoculated with *S. cerevisiae* strain α'1 (2×10^6 cells/plate). Plates were incubated for 2–3 days at 25 °C and non-growth zones were measured.

To determine the effect of pH on killing activity, MBA plates were adjusted to appropriate pH values and seeded with the *S. cerevisiae* strain α'1 (2×10^6 cells/plate). The sample of 100 µL of F_2-$K2_b$ particles or free K2 protein (4.0 µg/mL) was added to 10 mm wide wells formed in the agar layer. Plates were incubated for 2–3 days at 25 °C and non-growth zones around the wells were then measured and the activity was expressed in arbitrary units (Gulbiniene et al., 2004). The experiment was repeated five times and the data were represented as mean ± SD (U/mL).

To analyse the stability of encapsulated K2 toxin (F_2-$K2_b$) or free K2 toxin at the concentration of 4.0 µg/mL, the samples were incubated at 4 °C and 22 °C, and the aliquots were taken at different time points and subjected to agar-diffusion assay analysis. A volume of 20 µl was spotted on the MBA plate adjusted to the appropriate pH value and seeded with the sensitive *S. cerevisiae* strain α'1 (2×10^6 cells/plate). Inhibition zones were determined in triplicate plates after 2–3 days of incubation at 25 °C. The mean of residual toxin activity was compared with the initial activity and expressed as a percentage.

Table 1
Size of K2 toxin–loaded fucoidan particles at different pH values.

Samples	Hydrodynamic diameter, nm			
	F_1-$K2_a$	F_1-$K2_b$	F_2-$K2_a$	F_2-$K2_b$
		pH 3.5		
Control	177.23± 41.94	242.55 ± 58.62	179.23 ± 42.16	251.00 ± 12.73
Fresh particles	230.83 ± 22.60	237.20 ± 57.98	257.33 ± 57.67	224.87 ± 34.93
Particles after a month at 4 °C	150.43 ± 63.56	206.20 ± 1.41	173.25 ± 51.69	236.6 ± 67.10
		pH 4.2		
Control	173.97 ± 36.84	182.50 ± 13.15	175.23 ± 13.51	185.75 ± 2.19
Fresh particles	284.70 ± 7.50	235.37 ± 50.41	260.10 ± 1.84	259.80 ± 9.48
Particles after a month at 4 °C	234.85 ± 75.73	254.90 ± 2.97	188.37 ± 47.12	143.30 ± 27.58
		pH 4.8		
Control	192.90 ± 11.43	269.73 ± 27.91	317.63 ± 58.68	291.90 ± 12.41
Fresh particles	202.70 ± 20.13	230.35 ± 23.41	198.65 ± 28.35	180.85 ± 1.77
Particles after a month at 4 °C	187.50 ± 13.15	177.77 ± 25.89	96.50 ± 31.96	297.47 ± 97.35

2.6.2. Plate counting assay

A direct survival assay was used to analyse the antifungal activity of K2 toxin-loaded-fucoidan nanoparticles. Yeast cells grown overnight were collected by centrifugation at 5000 × *g* for 5 min, washed 3 times with 1 mol/L of sorbitol solution, and resuspended in 1 mol/L of sorbitol solution to a final concentration of approximately 1×10^7 cells/mL. Fifteen microlitres of yeast cell suspension were mixed with 85 µl of 1 mol/L of sorbitol solution, and 100 µl of toxin-loaded F_2-$K2_b$ particles (or free fucoidan at a concentration of 0.4 mg/mL) were added. In control samples, the toxin particles were replaced by 1 mol/L sorbitol solution. The samples were incubated at room temperature (22 °C) for 6 and 24 h. Serial dilutions were performed in sterile 1 mol/L of sorbitol solution, and 50 µL of each solution was spread onto YPD-agar plates following the 2 days incubation at 30 °C. The viable cells were counted and presented as a percentage of the total population. The average number of viable yeast cells in the control sample at each time point was treated as 100 %.

2.7. Statistical analysis

Data are expressed as the mean ± standard deviation of three independent experiments unless otherwise specified. The plate counting assay data were analysed and boxplots were generated using the ggplot2 package (Wickham, 2016) in R software (R Core Team).

3. Results and discussion

3.1. Preparation and physicochemical characterization of particles

For the encapsulation of the K2 type toxin purified from *S. cerevisiae* strain M437, the formation of a polyelectrolyte complex was applied. Fucoidan, an anionic sulphated polysaccharide, is a strong polyelectrolyte with a pK_a value of 1.0–2.5 (Barbosa et al., 2019). The complexes were formed at three different pH values, i.e., pH 3.5, 4.2, and 4.8. The choice of pH values was limited by the inactivation of the K2 toxin at slightly acidic and neutral pH values (Lukša, Serva & Servienė, 2016) and the isoelectric point (pI) of the toxin. As previously reported, the pI of the K2-type toxin of *S. cerevisiae* is equal to 4.5 (Pfeiffer & Radler, 1984). On the other hand, considering the different media of possible particle application in food, it was necessary to choose different pH values. The complexes were prepared at two different concentrations of fucoidan and K2 toxin, as mentioned in Section 2.4.

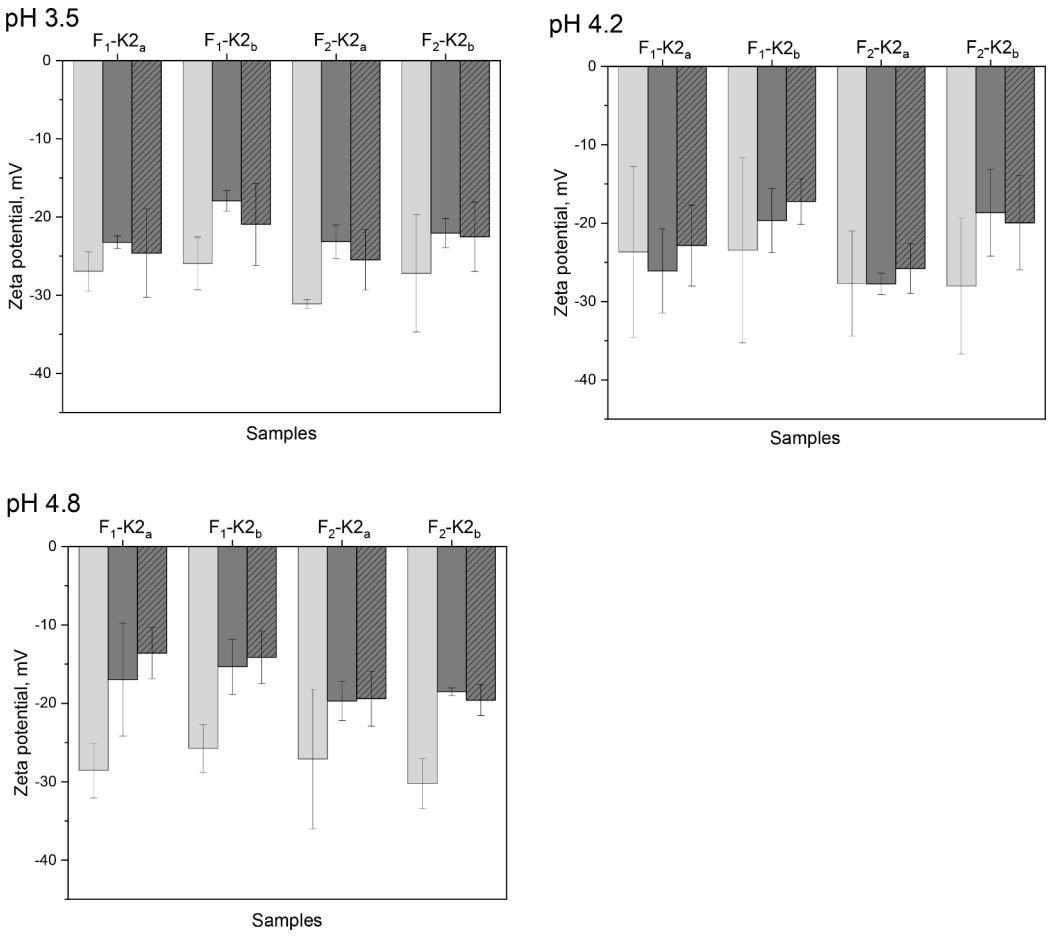

Fig. 1. Zeta-potential of K2 toxin-loaded fucoidan particles at different pH values. Control (fucoidan) - , fresh particles - , particles after one month storage at 4 °C - .

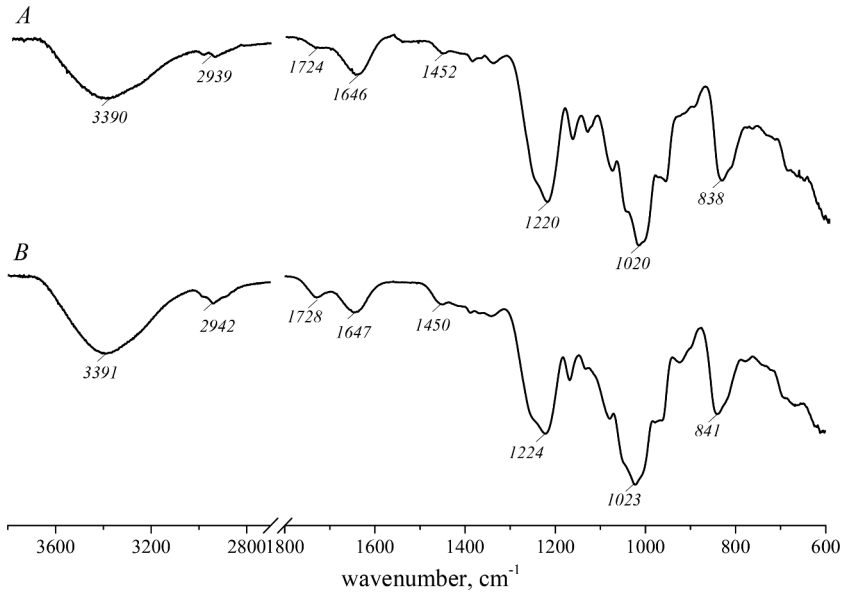

Fig. 2. FT-IR spectra of fucoidan (A) and K2 toxin–fucoidan complex (B).

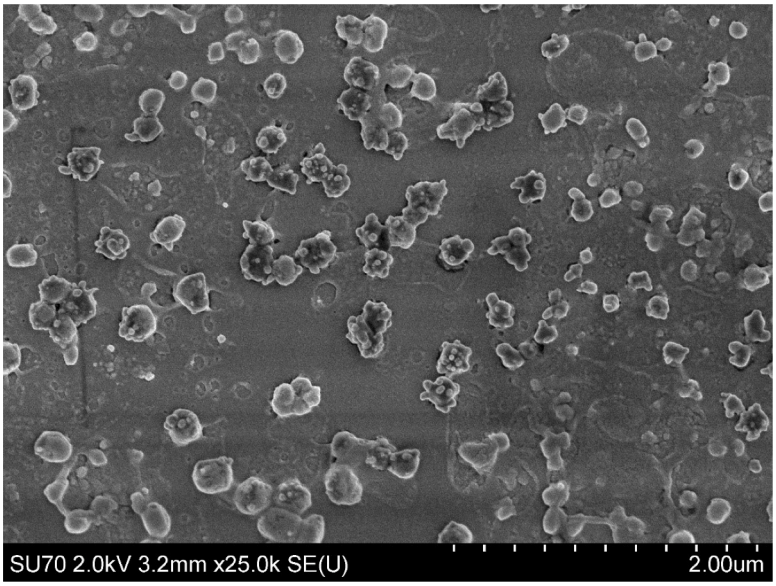

Fig. 3. SEM image of K2 toxin-loaded fucoidan particles.

Table 1 shows the size of the fresh particles and after storage for one month at 4 °C. As can be seen, the size of the complexes ranged from 180 to 280 nm. In some cases, especially at pH 4.2 and 4.8, the size of the particles tended to decrease after one month of storage. At pH values close to the pI of the protein, electrostatic interactions are not the dominant driving force. Protein-polysaccharide complexation also depends on other interactions such as hydrogen bonding and hydrophobic forces, and the flexibility of macromolecules is an important factor for such interactions (Wei & Huang, 2019; Turgeon, Beaulieu, Schmitt & Sanchez, 2003; Schmitt, Sanchez, Desobry-Banon & Hardy, 1998). The formation of fucoidan nanocomplexes with other proteins such as ovalbumin and β-lactoglobulin has also been observed under these conditions (Burova et al., 2022, 2023). The decrease in complex size could be also related to conformational rearrangements of the macromolecules. The particles had a negative surface charge of approximately −20–25 mV (Fig. 1). In most cases, the complexation of fucoidan with the K2 toxin only slightly increased the zeta potential. This may be due to the large differences in protein and polysaccharide concentrations, which range from 25 to 154 times. K2 toxin has strong killing activity, but *S. cerevisiae* strains secrete negligible amounts of the killing protein and its purification is quite difficult (Pfeiffer & Radler, 1984). Nevertheless, the particles obtained were active as described below.

The obtained FT-IR spectra of fucoidan and K2 toxin-loaded fucoidan particles are shown in Fig. 2.

The fucoidan spectrum shows two typical characteristic absorption bands at 1220 cm^{-1} corresponding to the symmetric stretching vibration of S═O and at 838 cm^{-1} corresponding to the stretching vibration of C—O-S (Zayed et al., 2016, 2023). Additionally, the common characteristic vibration bands for polysaccharides are found at 3390 cm^{-1} corresponding to the stretching of hydroxyls and bound water, and at 2939 cm^{-1} associated with the stretching of aliphatic C—H. The band at 1646 cm^{-1} and its shoulder at 1724 cm^{-1} are assigned to the structural water deformation band and the C═O stretching of the *O*-acetyl groups in some fucose residues, respectively (Dong et al., 2019; Florez-Fernandez et al., 2023; Synytsya et al., 2010). The absorption band at 1452 cm^{-1} is associated with symmetric CH_3 deformations of the fucose 6C methyl group (Dorschmann, Kopplin, Roider & Klettner, 2023). The intense band centered at 1020 cm^{-1} is a typical band of polysaccharides observed due to the C—OH, C—C, and C—O-C elongation vibrations (Gomez-Ordonez & Ruperez, 2011). After complexation, the bands at 1220 cm^{-1} and 838 cm^{-1} assigned to the sulphate group of fucoidan shift to 1224 cm^{-1} and 841 cm^{-1}, respectively. The shift indicates the electrostatic interaction between negatively charged sulphate groups and positively charged protein amino acid residues. As previously reported, the blue shift of both the S═O and the C—O-S stretching was observed due to the complexation of heparin with chitosan or *N,N,N*-trimethyl chitosan (Martins, Piai, Schuquel, Rubira & Muniz, 2011).

The Raman spectra of fucoidan and K2-toxin loaded fucoidan particles are shown in Fig. S1.

The Raman spectrum of fucoidan contains vibration bands that are characteristic of sulphated polysaccharides. The bands observed at 1267 cm^{-1} and 1054 cm^{-1} are associated with asymmetric and symmetric O═S═O stretching of the sulphate ester group. The band at 824 cm^{-1} is attributed to bending vibrations of C—O—S in the fucose ring (Dorschmann, Kopplin, Roider & Klettner, 2023; Marinval et al., 2016; Synytsya et al., 2010). After K2 toxin-fucoidan complexation, the bands at 1054 cm^{-1} and 824 cm^{-1} shift to 1050 cm^{-1} and 846 cm^{-1}, respectively, indicating protein-polysaccharide interaction. In addition, the sample of the complex was sensitive to laser power, resulting in the Raman spectrum of the complex having a poor signal-to-noise ratio. As previously reported by Ptak et al. (2021), Maillard reaction products could be formed during heating and have a negative effect on the spectrum.

UV–vis spectra of the samples were also recorded (Fig. S2). The shift of the peak in the spectrum of the K2 toxin-fucoidan complex was observed compared to the spectrum of the free toxin.

The SEM image shows that the particles are irregular in shape. It can be seen that some of them have aggregated during the preparation of the sample and their size is larger than that found by the dynamic light scattering method (Fig. 3).

Natural polysaccharide-based polyelectrolyte complexes have been used to develop delivery systems for bioactive compounds, including antimicrobial peptides. Various polysaccharides such as pectin (Krivorotova et al., 2016), carboxymethylcellulose (Celen, Anumudu, Miri, Onyeaka & Fernandez-Trillo, 2023), gum Arabic (Gong et al., 2018), or soluble soybean polysaccharide (Luo et al., 2019) have been shown to be suitable nanocarriers of nisin through polyion complex formation. Recently, the fucoidan-nisin complex has been developed and its activity has been demonstrated against Gram-positive bacteria (Gruškienė et al., 2024). The study shows that the simple and inexpensive complexation method using fucoidan as a carrier can be applied not only for antimicrobial peptides but also for larger antimicrobial proteins such as the K2 toxin.

Table 2
Killing activity of K2 toxin and K2 toxin-loaded fucoidan particles at different pH values [a].

Killing activity, U/mL		
pH	K2	F_2-$K2_b$
3.5	120.2 ± 24.9	131.8 ± 24.9
4.2	158.5 ± 26.1	144.5 ± 17.9
4.8	87.1 ± 11.3	95.5 ± 19.2

[a] The concentration of K2 protein was 4.0 μg/mL. For F_2-$K2_b$ preparation, the final concentrations of K2 protein and fucoidan were 4.0 μg/mL and 0.4 mg/mL, respectively.

3.2. Killing activity of K2 toxin-loaded fucoidan particles

The killing activity of toxin-loaded fucoidan nanoparticles was tested against *S. cerevisiae* α'1 strain, which is known to be sensitive to the action of the K2 protein (Lukša, Serva & Servienė, 2016). As can be seen, the K2 toxin complexed with fucoidan retained its biological activity (Table 2) and its activity was pH dependent. The highest was found at pH 4.2. The activity of the encapsulated toxin decreased slightly at pH 3.8 while at pH 4.8 only about 66 % of the killer protein activity remained compared to the optimal conditions (pH 4.2). The activity of K2 toxin-loaded fucoidan nanoparticles was comparable to that of free K2 under the corresponding pH conditions. As previously found, the K2 toxin has the most acidic pH optimum compared to other extrachromosomally encoded killer proteins secreted by *S. cerevisiae* (Lukša, Serva & Servienė, 2016). The K1 killer toxin has its maximum activity in a very narrow pH range of 4.5–4.6 (Kurzweilova & Sigler, 1993). The pH optimum of the K28 toxin is at pH 5.8 (Schmitt & Tipper, 1990). The Klus toxin shows the highest activity at pH 4.0–4.7 (Rodriguez-Cousino et al., 2011).

The complexation of the K2 toxin with fucoidan increased its stability (Fig. 4). In general, K2 toxin inactivation is pH and temperature-dependent (Lukša, Serva & Servienė, 2016). The stabilisation effect of fucoidan was most evident at room temperature. As can be seen, the purified free K2 toxin loses 50 % of its killing activity in about two weeks and four days at pH 3.5 and 4.2, respectively, whereas the half-times of inactivation of the encapsulated toxin were approximately one month and twenty-four days, respectively. As the optimal temperature required for K2 killing activity is in the range of 20–25 °C (Lukša, Serva & Servienė, 2016), the stabilisation of the K2 toxin extends the duration of its functionality at room temperature increasing its applicability in the food industry. At pH 4.8 and room temperature, the free K2 toxin is very unstable and loses its activity within one day. However, even at pH 4.8 complexation with fucoidan extends the period to three days. At 4 °C, the K2 toxin is quite stable and even at pH 4.8 retains 70 % of its killing activity for more than a month.

The stabilisation of proteins by sugars is a well-known phenomenon. The osmolytes sucrose and trehalose are the most commonly used sugars for this purpose. They protect proteins from thermal and chemical denaturation and from losing biological functions (Ajito, Iwase, Takata & Hirai, 2018). Recently, natural polysaccharides have been proposed as an effective biotechnological tool for protein stabilisation. Their efficacy has been demonstrated for fungal cellobiose dehydrogenase using microbial polysaccharides (Sulej et al., 2021). In this study, the ability of the seaweed polysaccharide fucoidan to stabilise the protein with an antifungal killing activity has been shown.

Furthermore, to test the efficacy of the K2 toxin-loaded fucoidan particles in potential applications, the killing activity against brewing and winemaking yeast contaminants of the *Candida, Pichia, Torulaspora*, and *Saccharomyces* genera was analysed using the direct survival assay (Fig. 5).

The most effective inhibitory activity of the particles was detected against *Saccharomyces cerevisiae* yeast, which is frequently associated with wine refermentation, altering its chemical composition, and contaminating bread and its products (Hernandez et al., 2018). The viability of other yeast strains tested, such as *P. membranifaciens, P. anomala, P. holsti*, and *T. delbrueckii*, was also reduced under the action of K2 toxin-loaded fucoidan particles, but to a lesser extent. The tested yeasts are known to form a film on the surface of bulk wine and affect the growth of the primary winery microflora; therefore, they can be used as indicators for spoilage monitoring (Loureiro & Malfeito-Ferreira, 2003; Hernandez et al., 2018). Besides, the viability of potential human pathogen *C. albicans*, often associated with the food and beverage industry, was also reduced after the treatment with encapsulated K2 protein. As shown in Fig. 5, fucoidan alone also had some antifungal activity against the microorganisms tested. These data add to the knowledge of the antimicrobial properties of fucoidan.

4. Conclusion

Fucoidan was shown to be a suitable carrier for the killer toxin K2. The particles were prepared by the low-cost complexation method and characterised using FT-IR, Raman, and UV–vis spectroscopy, and scanning electron microscopy. Dynamic light scattering showed that the particles ranged in size from 180 to 280 nm and were stable for at least one month at 4°C. The encapsulated K2 toxin retained its antimicrobial activity. In addition, complexation with fucoidan increased the stability of the K2 toxin at room temperature. Formulation of the K2 toxin with fucoidan increases its biotechnological potential. The killing activity of encapsulated K2 toxin against spoilage yeasts in wine production and the increased stability make K2 toxin a new-generation antimicrobial agent. This opens up possibilities for its use in the food and beverage industry.

CRediT authorship contribution statement

Rūta Gruškienė: Writing – original draft, Validation, Investigation, Conceptualization. **Aistė Galinskaitė:** Investigation. **Tatjana Kavleiskaja:** Visualization, Investigation. **Iglė Vepštaitė-Monstavičė:** Visualization, Investigation. **Elena Servienė:** Writing – original draft,

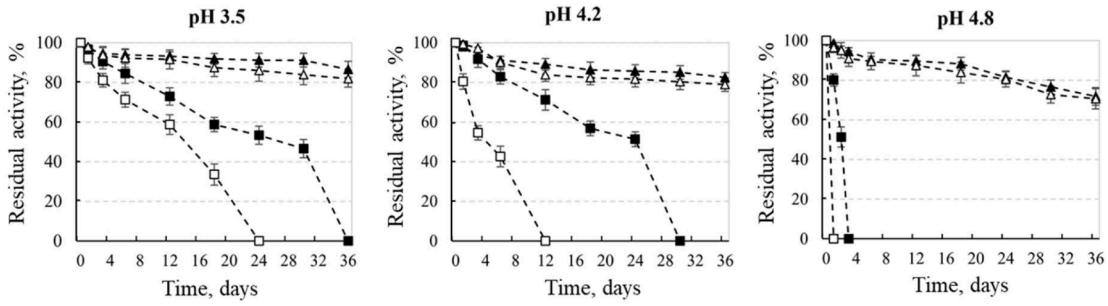

Fig. 4. Stability of free (□, Δ) and encapsulated K2 toxin (■, ▲) at different pH values; □, ■, 22 °C; Δ, ▲, 4 °C. The concentration of free K2 toxin was 4.0 μg/mL. For encapsulation, the final concentrations of K2 toxin and fucoidan were 4.0 μg/mL and 0.4 mg/mL, respectively.

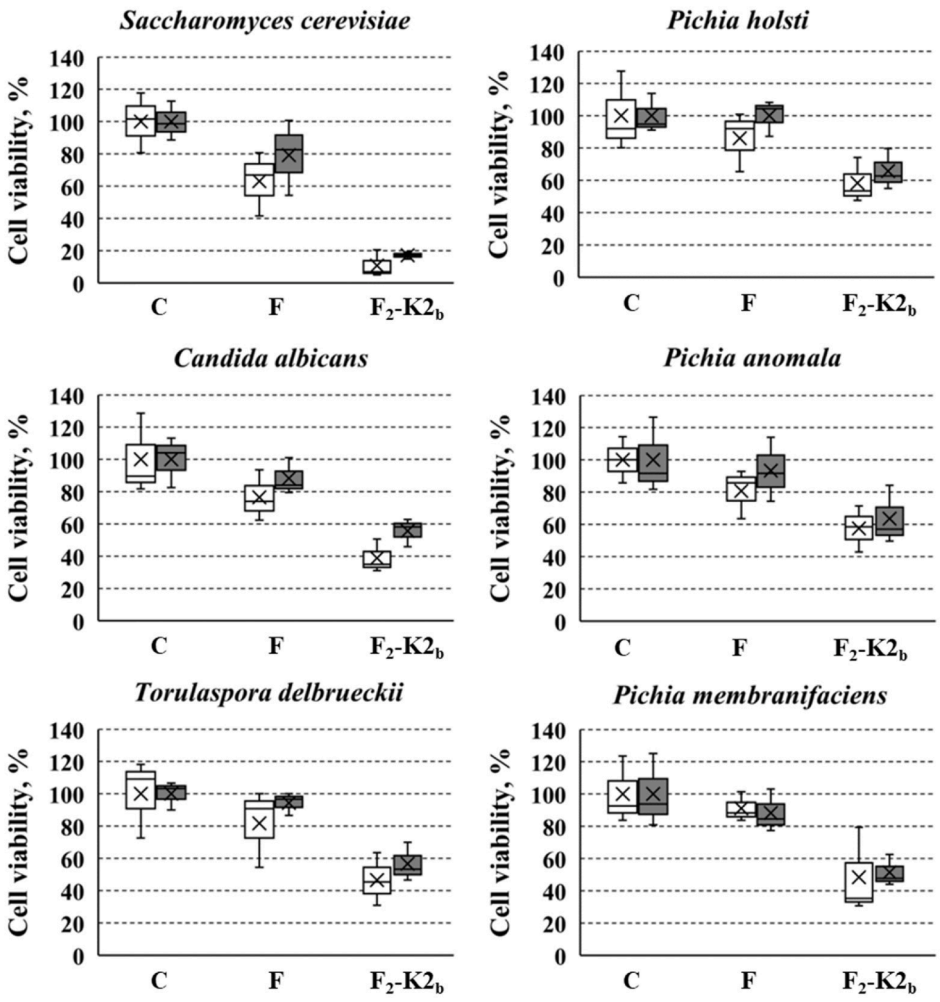

Fig. 5. Boxplot representation of yeast survival under the action of K2 toxin-loaded particles. C – yeast cells only; F – yeast cells mixed with fucoidan solution of 0.4 mg/mL; F_2-$K2_b$ – yeast cells incubated with K2 toxin-loaded fucoidan particles which were prepared at the final concentration of K2 toxin and fucoidan of 4.0 μg/mL and 0.4 mg/mL, respectively. White bars – cells viability after 6 h treatment; grey bars – 24 h treatment. Horizontal black line – median; whiskers – minimal and maximal values; cross – mean.

Validation, Conceptualization. **Jolanta Sereikaitė:** Writing – review & editing, Supervision, Project administration, 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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpta.2024.100521.

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