Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 6, 2014

Original Article

THE EFFECT OF ENCAPSULATION ON THE *IN VITRO* ANTI-OXIDATIVE ACTIVITY OF RUTIN

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Received: 08 May 2014 Revised and Accepted: 06 Jun 2014

ABSTRACT

Objective: This study was investigating the effect of encapsulation on the *in vitro* anti-oxidative activity of rutin.

Methods: Rutin encapsulated in non-amidated low methoxyl pectin bead in various formulations with or without sorbitol and/or sodium bicarbonate ($NaHCO_3$) were prepared as well as *in vitro* anti-oxidative assays including 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging, chelating, lipid peroxidation inhibition and 2,2'-azinobis-3-ethylbenthiazoline-6-sulfonic acid (ABTS) radical cation decolorization were investigated.

Results: The rutin encapsulated in 3NA15Sor1Bica (3% non-amidated LMP with 15% sorbitol and 1% $NaHCO_3$) beads showed anti-oxidative activity higher than the other formulations on DPPH radical scavenging (84.00 ± 4.47%), chelating (74.11 ± 4.29%) and ABTS radical cation decolorization (80.04 ± 7.93%) activities.

Conclusion: The results from this study can be applied for the further development of the encapsulation formulation by adding the suitable proportion of sorbitol and $NaHCO_3$ in order to maintain the activities of encapsulated compounds.

Keywords: Low methoxyl pectin, Rutin, Anti-oxidative activity; Encapsulation.

INTRODUCTION

Active agents such as antioxidants are not only protect the manufacture of products but also exhibit many beneficial effects on human health [1]. Every year, new compounds with antioxidant capability and how to protect them have promoted interest. Phenolic compounds are the most sought by both food and pharmaceutical industries for their antioxidant property that neutralize the action of free radicals, avoid or retard lipid peroxidation processes and cell damage [2,3]. It has been estimated that 2% of the oxygen consumed by a normal organism contributes to the formation of reactive oxygen species (ROS) of which various are free radicals. When ROS generation surpasses the organism's antioxidant defenses, independently of the mechanism (ultraviolet radiation, environmental pollution, strenuous physical activity, or others), biological structures suffer damage caused by chemical lesions in an oxidative stress process which is involved in developing degenerative pathologies and low plasmatic antioxidant concentrations [4]. The flavonoids rutin (quercetin-3-*O*-rutinoside) is polyphenolic compounds found in several medicinal plants, vegetables, fruits, herbs, leaves and seeds [5,6]. Rutin is a bioflavonoid and antioxidant that presents important properties in human health like its significant scavenging properties on oxidizing species such as hydroxyl, superoxide and peroxyl radicals [7].

Microencapsulation is an alternative technology to stabilize stress factors and protect the pharmaceutical compounds, food ingredients or additives, which include environmentally sensitive bioactive principles in protective matrices to enhance their functionality and life span. These sensitive compounds are preserved by protective matrices allowing them to be added to pharmaceutical dosage forms or foods and increase their functionality [8]. Pectin, one of protective matrix, is a naturally non-toxic biopolymer that is finding increasing applications in the pharmaceutical and biotechnology industries. It has also been used successfully for many years in the food and beverage industries as thickening and gelling agents. Pectin has several unique properties that have enabled it to be used as a matrix for the entrapment and/or delivery of a variety of drugs, proteins and cells [9]. Thus, rutin which is the light sensitive antioxidant could be encapsulated by using pectin as a protective matrix in order to maintain its functionality. However, there are no data showing about any activities of encapsulated rutin in low methoxyl pectin beads on anti-oxidative activity. In this present study, rutin encapsulated in non-amidated low methoxyl pectin bead in various formulations with or without sorbitol and/or sodium bicarbonate were prepared as well as *in vitro* anti-oxidative assays including 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging, chelating, lipid peroxidation inhibition and 2,2'-azinobis-3-ethylbenthiazoline-6-sulfonic acid (ABTS) radical cation decolorization were investigated.

MATERIALS AND METHODS

Materials

Non-amidated low methoxy pectin (LMP) (Unipectine OF300C; DE = 30% and DA = 0%) were purchased from Cargill™ (Saint Germain, France). Rutin hydrate, L-(+)-ascorbic acid (vitamin C), butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picryhydrazyl radical (DPPH), ammonium thiocyanate (NH_4SCN), ethylenediaminetetraacetic acid (EDTA), ferrozine, ferric chloride ($FeCl_2$), ABTS (2,2'-azinobis-(3-ethylbenthiazoline-6-sulfonic acid), potassium persulfate and sodium bicarbonate ($NaHCO_3$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sorbitol pure anhydrate was purchased from Cooperation Pharmaceutique Francaise (Melun, France). Absolute ethanol was purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals and reagents were analytical grade.

Preparation of low methoxyl pectin (LMP) beads by encapsulator

The ionotropic gelation technique was used for rutin encapsulating in the beads. Four formulations of rutin beads including 3NA (3% non-amidated LMP), 3NA15Sor (3% non-amidated LMP with 15% sorbitol), 3NA1Bica (3% non-amidated LMP with 1% $NaHCO_3$) and 3NA15Sor1Bica (3% non-amidated LMP with 15% sorbitol and 1% $NaHCO_3$) were prepared as previously described [10].

Anti-oxidative assays

DPPH radical scavenging activity

The DPPH radical scavenging activity of all beads was determined by a modified method previously described [11]. Briefly, the five serial concentrations of rutin beads (0.05-0.40 µg/mL of encapsulated rutin) with 50 µL of distilled water and 50 µL of DPPH ethanolic solution were put into each well of a 24-well plate (Nalge Nunc International, NY, USA). The reaction mixture was allowed to stand for 30 min at 25 ± 2°C, after that the reacted solution was separated,

put in to 96-microplate and measured the absorbance at 515 nm by a well reader (Bio-Rad, model 680 microplate reader, USA) against blank (ethanol). Ascorbic acid, BHT and non-encapsulated rutin (0.001-10 mg/mL) were used as positive controls. The experiments were done in triplicate. The percentages of DPPH radical scavenging activity were calculated [Scavenging (%) = (Abs $_{control}$ – Abs $_{sample}$)/Abs $_{control}$ × 100%]. The histogram of the percentages of DPPH radical scavenging activity of beads at 0.4 µg/mL and the standard antioxidants was presented.

Chelating activity

The Fe^{2+} chelating ability of the beads was measured by the ferrous iron–ferrozine complex method [12]. Briefly, the reaction mixture containing 2 mM $FeCl_2$ (10 µL) and 5 mM ferrozine (10 µL) and the five serial concentrations of beads (0.05-0.40 µg/mL of encapsulated rutin in 100 µl distilled water) were mixed in a 24-well plate, then incubated for 10 min at 25 ± 2°C after that the reacted solution was separated and put in to 96-microplate. The absorbance was recorded by a well reader at 570 nm. The absorbance of the control was determined by replacing the beads with ethanol. EDTA (0.001-10 mg/mL) was used as a positive control. The experiments were done in triplicate. The ability of the sample to chelate ferrous ion was calculated [Chelating effect (%) = [(Abs $_{control}$ – Abs $_{sample}$)/Abs $_{control}$ × 100%]. The histogram of the chelating effect percentages of beads at 0.4 µg/mL and the standard EDTA was presented.

Lipid peroxidase inhibition activity

The lipid peroxidase activity of rutin beads were assayed by the modified Ferric-thiocyanate method [13,14]. An amount of 100 µL distilled water and five serial concentrations of beads (0.05-0.40 µg/mL of encapsulated rutin) was added to 50 µL of linoleic acid in 50% (v/v) DMSO in each well of a 24-well plate. The reaction was initiated by the addition of 50 µL of NH_4SCN (5 mM) and 50 µL of $FeCl_2$ (2 mM). The mixture was incubated at 37 ± 2°C for 1 h. During the oxidation of linoleic acid, peroxides are formed leading to the oxidation of Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate which can be detected at 490 nm (the reacted solution was separated, put in to 96-microplate before measurement). The distilled water without the bead sample was used as negative control. α-Tocopherol (at 0.001-10 mg/mL) was used as a positive control. All experiments were performed in triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated as the following: Inhibition of lipid peroxidation (%) = (Abs $_{control}$ – Abs $_{sample}$)/Abs $_{control}$ × 100%. The sample concentration providing 50% inhibition of lipid peroxidation (IC_{50}) was calculated from the graph plotted between the percentages of lipid peroxidation inhibition and the sample concentrations.

ABTS radical cation decolorization assay

ABTS can produce stable free radicals, which are decolorized into their non-radical form when reacting with antioxidants. The method for determining ABTS radical scavenging activity was modified from Re et al. [15]. Briefly, $ABTS^{\cdot+}$ was generated by oxidation of 7 mM ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) and stored in a dark place at room temperature (25 ± 2°C) for 12-16 h. Then ABTS stock solution was diluted with deionized water to obtain ABTS working solution. The reactions between 2 mL of ABTS working solution and five serial concentrations of rutin beads (0.05-0.40 µg/mL of encapsulated rutin) were initiated and stored at 37 ± 2°C until the reaction was completed. After that, the remained beads were removed and measured the absorbance at 734 nm against a blank (ethanol). Ascorbic acid (0.001-10 mg/mL) was used as positive controls. The experiments were done in triplicate. The IC_{50} value which was the concentration of the sample that scavenged 50% of the ABTS radical was determined. The percentages of ABTS radical scavenging activity were calculated [ABTS scavenging (%) = (Abs $_{control}$ – Abs $_{sample}$)/Abs $_{control}$ × 100%]. The histogram of the percentages of ABTS radical cation decolorization of beads at 0.4 µg/mL and the standard ascorbic acid was presented.

Statistical analysis

The results were presented as the mean of three independent experiments and the standard deviation (SD). ANOVA was used for the analysis of the test results at the significance level of *p*-value <0.05.

RESULTS AND DISCUSSION

Beads preparation and previous characterization studied

The beads were formed by ionotropic gelation mechanism in which intra-molecular crosslinks were formed between the negatively charged carboxyl groups of low methoxy pectin (LMP) and the positively charged counter ion (Ca^{2+}) and rutin particles were encapsulated. From our previous results, all beads were spherical shape. The high encapsulation efficiency was observed in 3NA and 3NA15Sor beads (95.58 ± 1.36 and 94.43 ± 0.79%, respectively). The formulation composed of $NaHCO_3$ (3NA1Bica and 3NA15Sor1Bica) revealed the lower rutin encapsulation efficiency (80.40 ± 0.84 and 79.77 ± 1.45%, respectively) because of $NaHCO_3$ in their formulations. The order of rutin released from formulations was followed 3NA15Sor > 3NA15Sor1Bica > 3NA > 3NA1Bica in phosphate buffer (PB) pH 7.4. The pectinate beads containing sorbitol (3NA15Sor) showed the best release efficiency and swelling behaviour whereas the beads containing sorbitol and $NaHCO_3$ (3NA15Sor1Bica) showed burst release with less lag time [16].

Table 1: Previous studied on Young's modulus value, encapsulation efficiency, percentages of rutin released at 1 h and lag time of 2% w/v rutin LMP bead formulations release profile

Sample	3NA	3NA15Sor1Bica	3NA15Sor	3NA1Bica
Young's modulus (Pa) of gel	1124.20 ± 462.38	171.34 ± 79.03	107.64 ± 21.93	1004.14 ± 81.00
Encapsulation efficiency (%)	95.58 ± 1.36	79.77 ± 1.45	94.43 ± 0.79	80.40 ± 0.84
Rutin release at 1 h in PB (%)	35.87 ± 4.00	54.76 ± 2.33	66.85 ± 2.10	6.87 ± 1.12
Lag time of rutin release profile (min)	30	5	20	40

DPPH radical scavenging activity

Figure1 demonstrated the percentages of DPPH radical scavenging activity of the beads at 0.4 µg/mL (calculated from amount of the encapsulated rutin). The standard antioxidants (ascorbic acid, BHT and non-encapsulated rutin at 0.4 µg/mL) gave the scavenging activity of 92.03 ± 1.33, 88.96 ± 2.77 and 87.41 ± 0.17%, respectively (IC_{50} values cannot be calculated because the lowest concentration, 0.05 µg/mL, gave scavenging activity more than 50%). The beads composed of 15% sorbitol and 1% $NaHCO_3$ (3NA15Sor1Bica) exhibited higher DPPH radical scavenging activity (84.00 ± 4.47%) than the others which were 3NA1Bica (23.04 ± 8.89%), 3NA15Sor (17.80 ± 1.74%) and 3NA (15.54 ± 8.97%), respectively. DPPH is a stable free radical and accepts electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance induced by antioxidants. It is visually noticeable as discolouration from purple to yellow. The effect of rutin on DPPH radical scavenging is thought to be due to its hydrogen donating ability [17-19]. The result also indicated that encapsulated rutin in 3NA15Sor1Bica LMP bead formulation did not effected to the DPPH radical scavenging activity (84.00 ± 4.47%) when compared with non-encapsulated rutin which gave scavenging activity 87.41 ± 0.17% (p<0.05). The synergistic effect of sorbitol (15Sor) which promoted the bead softness and the burst release property of $NaHCO_3$ (1Bica) will be correlated for this activity. However, encapsulated rutin in 3NA1Bica, 3NA15Sor and 3NA bead

formulations gave less scavenging activity than non-encapsulated rutin. This may be due to the shielding effect of LMP bead together with the bead hardness and lack of initial release property. Thus, rutin may not be released from the beads. Therefore, the potent DPPH radical scavenging activity of rutin still remained even if it was encapsulated in 3NA15Sor1Bica beads.

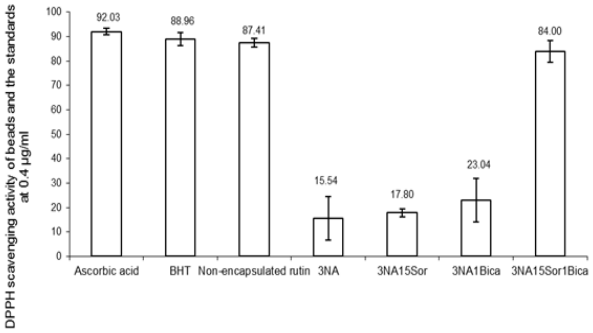

Fig. 1: Comparison of the percentages of DPPH radical scavenging activity of beads and the standard antioxidants at 0.4 µg/mL

Chelating activity

Figure2 showed the percentages of chelating activity of the beads at 0.4 µg/mL (calculated from amount of the encapsulated rutin). The standard compounds (EDTA and non-encapsulated rutin at 0.4 µg/mL) gave the chelating activity of 87.74 ± 1.00 and 78.58 ± 7.35%, respectively (IC_{50} values cannot be calculated because the lowest concentration, 0.05 µg/mL, gave chelating activity more than 50%). The bead composed of 15% sorbitol and 1% $NaHCO_3$ (3NA15Sor1Bica) also exhibited higher chelating activity (74.11 ± 4.29%) than the others which were 3NA1Bica (66.63 ± 3.49%), 3NA15Sor (40.28 ± 8.31%) and 3NA (26.63 ± 2.37%), respectively. Flavonoids exert their antioxidant activity by scavenging reactive oxygen species (ROS) and preventing ROS formation with chelating transition metal ions such as iron and copper, which play vital roles in the initiation of free radical reactions [20]. Rutin has been reported its chelating activities in reducing Fe(II)-induced malondialdehyde formation in liposomes [21] and $FeSO_4$/cysteine-induced lipid peroxidation in rat liver microsomes [22]. This result has supported that rutin still showed chelating activity although it was encapsulated in LMP beads.

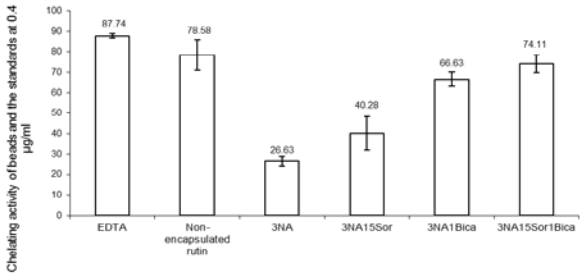

Fig. 2: Comparison of the percentages of chelating activity of beads and the standards at 0.4 µg/mL

Lipid peroxidation inhibition

Standard ascorbic acid and non-encapsulated rutin at 0.4 µg/mL gave the lipid peroxidation inhibition activity of 96.68 ± 2.38 and 63.14 ± 3.60% with IC_{50} value of 0.26 ± 0.04 and 0.38 ± 0.10 µg/mL, respectively. None of the rutin LMP beads indicated chelating activity except rutin encapsulated in 3NA15Sor1Bica which gave the lipid peroxidation inhibition activity of 61.09 ± 7.16% at 0.4 µg/mL (IC_{50} value of 0.46 ± 0.09 µg/mL). In fact, rutin was significantly effective inhibitor of iron ion-dependent lipid peroxidation system due to chelating iron ions with the formation of inert iron complexes unable to initiate lipid peroxidation [23]. However, encapsulated rutin in 3NA1Bica, 3NA15Sor and 3NA formulations exhibited no lipid peroxidation inhibition activity. This result can anticipate the strong effect of the encapsulation formulation on the anti-oxidative activity especially lipid peroxidation inhibition activity of rutin.

ABTS radical cation decolorization

ABTS and DPPH are the most popular assays, among others such as oxygen radical absorbance capacity (ORAC) and ferric reducing ability of plasma (FRAP) assays [24-26]. The ABTS assay is based on the generation of a blue/green $ABTS^{\cdot+}$ that can be reduced by antioxidants, whereas the DPPH assay is based on the reduction of the purple $DPPH^{\cdot}$ to 1,1-diphenyl-2-picryl hydrazine. These two assays are based on an electron transfer and involve reduction of a colored oxidant [27-28]. Thus, ABTS radical decolorization assay in this study was done in order to confirm the DPPH radical scavenging result. Figure3. demonstrated the percentages of ABTS radical decolorization activity of the beads at 0.4 µg/mL, calculated from amount of the encapsulated rutin. Ascorbic acid which was positive control gave the highest ABTS decolorization activity at 96.86 ± 2.77% with IC_{50} value of 0.08 ± 0.01 µg/mL. For the rutin LMP beads, the result of ABTS showed the same trend as DPPH assay. 3NA15Sor1Bica encapsulated with rutin exhibited higher activity (80.04 ± 7.93%) than the others, which were 3NA1Bica (56.32 ± 5.88%), 3NA15Sor (7.94 ± 4.78%) and 3NA (9.56 ± 1.11%), respectively. 3NA15Sor1Bica and 3NA1Bica gave the IC_{50} values of 0.28 ± 0.01 and 0.43 ± 0.04, respectively. The IC_{50} values of 3NA15Sor and 3NA cannot be calculated because the highest concentration (0.4 µg/mL) gave ABTS decolorization activity less than 50%.

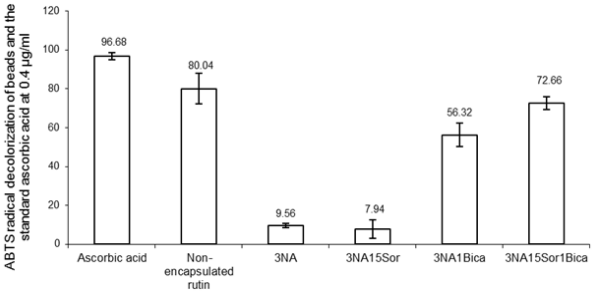

Fig. 3: Comparison of the percentages of ABTS decolorization activity of beads and the standard ascorbic acid at 0.4 µg/mL

CONCLUSION

This present study has demonstrated the *in vitro* anti-oxidative activity of various formulations of rutin encapsulated in low methoxyl pectin beads. Rutin which was encapsulated in 3NA15Sor1Bica (3% Non-amidated LMP with 15% sorbitol and 1% $NaHCO_3$) bead showed anti-oxidative activity higher than the other bead formulations on DPPH radical scavenging, chelating, lipid peroxidation inhibition and ABTS radical cation decolorization activities. The results from this study can be applied for the further development of the encapsulation by adding the suitable proportion of sorbitol and $NaHCO_3$ in the formulation in order to maintain the activities of encapsulated compounds. The further assesse the *in vivo* anti-oxidative activity in animal or human volunteer should be evaluated.

ACKNOWLEDGEMENTS

This work was supported by Faculty of Pharmacy and Chiang Mai University. The authors sincerely thank Prof. Dr. Aranya Manosroi, mentor, under the New Researcher Project 2013, Chiang Mai University, Thailand and Prof. Odile Chambin**,** Department of Pharmaceutical Technology, School of Pharmacy, Université de Bourgogne, 7 bd Jeanne d'Arc 21079 Dijon, France.

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