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Enzymatic deglycosylation of flavonoids in deep eutectic solvents-aqueous mixtures: paving the way for sustainable flavonoid chemistry

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ABSTRACT

The low solubility of glycosylated flavonoids represents a hurdle to conduct efficient enzymatic deglycosylations in aqueous media. To overcome this drawback, environmentally-unfriendly dimethylsulfoxide (DMSO) is typically used as co-solvent. Using a specific diglycosidase from *Acremonium* sp. DSM24697 for the deglycosylation of the rutinosylated flavonoid (hesperidin) as model reaction, this communication explores the use of (non-hazardous and biodegradable) deep eutectic solvents (DESs) as co-solvents in flavonoid biocatalysis. The enzymatic deglycosylation was observed when DES composed of choline chloride and glycerol or ethylene-glycol was used at proportions of up to 40% (DES-Buffer, v/v), displaying a promising framework to combine enhanced flavonoid solubilities and high enzymatic activities. The deglycosylation activity significantly increased when the single DES components – glycerol and ethylene-glycol – were added (e.g. 140% of enzyme activity at glycerol at 40% v/v), whereas deleterious effects were observed when choline chloride was solely added, presumably due to its chaotropic effect. Future research opportunities may be envisaged in the genetic design to evolve more robust biocatalysts, and in tailoring DES to deliver more enzyme-compatible solvents.

Flavanone glycosides are secondary metabolites that occur widely in citrus species, influencing citrus fruit production and processing. The deglycosylation of different flavonoid glycosides represents a necessary step in food technology for de-bittering and clarifying fruit juices [1]. Likewise, several articles have reported biological activities for diverse de-glycosylated flavanones [2,3]. Particularly hesperetin – the aglycone of flavonoid hesperidin (see Scheme 1) –, displays analgesic, anti-inflammatory, and antioxidant properties, and it can also be used as building block for the production of dyes and sweeteners [3]. Despite its importance, however, to date only few bioprocesses efficiently hydrolyzing hesperidin to afford hesperetin have been disclosed [4,5]. They typically involve sequential steps catalyzed by two mono-glycosidases (α-L-rhamnosidase (EC 3.2.1.40) and β-glucosidase (EC 3.2.1.21)), releasing rhamnose and glucose in each step, respectively. Remarkably, a single-deglycosylating-step procedure using the specific diglycosidase 6-*O*-α-rhamnosyl-β-glucosidase (EC 3.2.1.168) – directly rendering the disaccharide rutinose and hesperetin – has been recently described by our research group [6]. The enzyme is a fungal glycoprotein (Mw 42 kDa; pI 5.7) – produced by *Acremonium* sp. DSM24697 – reported to have a higher affinity to the substrate hesperidin (K_m 1.77 mM) in comparison with the substrate hesperidin methylchalcone (K_m 8.73 mM) [6]. This diglycosidase also enables the transglycosylation of rutinosyl units using the flavonoids hesperidin or hesperidin methyl-chalcone as rutinose donor [7].

To pave the way for an efficient biocatalytic process for hesperetin in particular – and with a broader perspective, for flavonoids in general –, the substrate solubilization in aqueous media represents a challenge, given the low solubility of flavonoids in such conditions. On this basis, the use of enzyme-compatible (and acceptable for food industry) co-solvents to enable proper dissolution of organic compounds would be of utmost importance. Yet, to our knowledge only dimethylsulfoxide (DMSO) has been assessed for enzyme catalysis in flavonoid-based reactions [7–10]. Albeit DMSO leads to excellent enzymatic activities and outstand-

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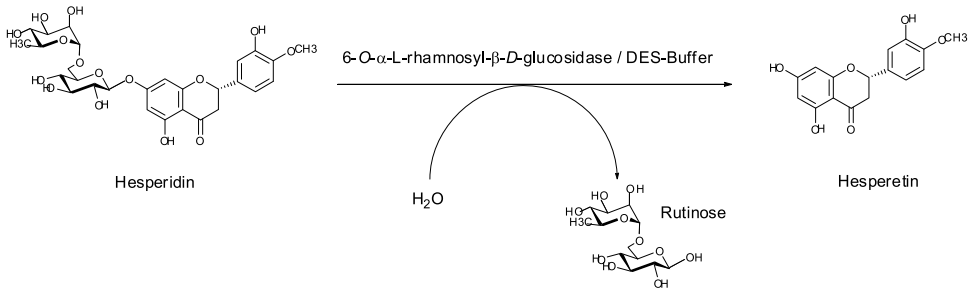

Scheme 1. One-step deglycosylation of hesperidin to afford hesperetin catalyzed by 6-*O*-α-rhamnosyl-β-glucosidase.

ing solubilizing properties, the use of DMSO poses considerable disadvantages regarding enzyme stability in flavonoid chemistry [9] as well as when scale-up and downstream processing steps are envisioned (e.g. wastewater formation, energy-demanding distillations, loss of products, etc.) [8,11,12]. In this respect, the introduction of less hazardous co-solvents to provide straightforward work-up procedures appears as a promising research line for flavonoid chemistry.

In the quest for novel tuneable alternatives, the use of deep-eutectic-solvents (DES) is envisaged. DES result from the complexation of quaternary ammonium salts (hydrogen acceptor: HA, e.g. choline chloride) with hydrogen bond donors (HBD) such as amines, amides, alcohols or carboxylic acids. The reduction of anion-cation electrostatic forces decrease the freezing point of the mixture rendering environmental friendly tuneable solvents [13–16]. Being in most cases non-hazardous and biodegradable, the use of DES for substrates of low water solubility and for controlling water concentration in reaction mixtures have endower their application in biocatalysis [17], with outstanding examples for hydrolases [18], lyases [19], and even whole-cells containing oxidoreductases [20]. To our knowledge the use of DES in glycoside hydrolases has not been assessed so far, with the only exception of genetically-designed cellulases performing cellulose hydrolysis in seawater and DES-aqueous mixtures [21], or, for ionic liquids, using some imidazolium-based solvents for disaccharide synthesis [22]. Actually, DES have been claimed as the "natural solvents" able to dissolve molecules in nature (e.g. flavonoids, with outstanding solubilities of 20–90 g Kg^{-1}) [23], with potential extractive features to purify them from plant extracts [24]. Conclusively, identifying enzyme-compatible DES that could also be efficiently applied for flavonoid chemistry would represent an important step for the future implementation of these technologies at industrial level on a sustainable manner. On this basis, in this communication the use of DES for the deglycosylation of hesperidin is reported for the first time.

The model reaction of study is depicted in Scheme 1. As prototypical DES for biocatalysis [25], choline chloride was chosen as the quaternary ammonium salt, and was combined with glycerol, ethylene-glycol and urea as hydrogen-bond donors (all of them at 1:2 mol:mol). The neat DES displayed excellent solubilizing capabilities for hesperidin, dissolving up to 90 mM, fully consistent with previous literature for DES and flavonoids [23,24], as well as for other ionic liquids [26,27].

In a first set of experiments, the enzymatic one-step deglycosylation of hesperidin was assessed at different DES-buffer proportions (v/v), using 6-*O*-α-rhamnosyl-β-glucosidase as the biocatalyst (Scheme 1). Results are depicted in Fig. 1. As it can be observed, DES displayed considerable effects on the enzymatic glycosidase activity. Especially when urea was used as hydrogen bond donor (ChCl:Ur DES), no enzymatic activities were already detected at DES proportions of ca. 40% (v/v), suggesting the unfolding of the enzyme. However, DES formed with polyols as hydrogen bond

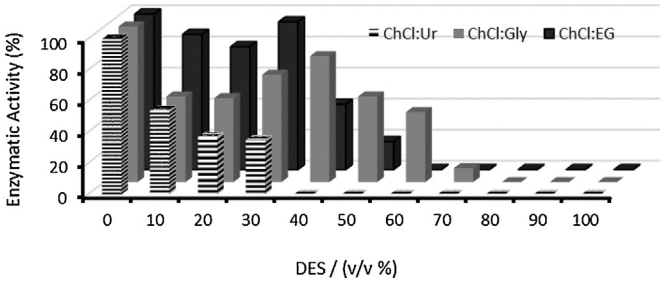

Fig. 1. Effect of DES concentration on the enzymatic activity for the deglycosylation of hesperidin by α-ramnosyl-β-glucosidase. See experimental part for reaction conditions. One hundred percent activity corresponded to 1.21 ± 0.17 U/mL, 1.30 ± 0.11 U/mL and 1.43 ± 0.10 U/mL for ChCl:EG, ChCl:Gly and ChCl:Ur respectively.

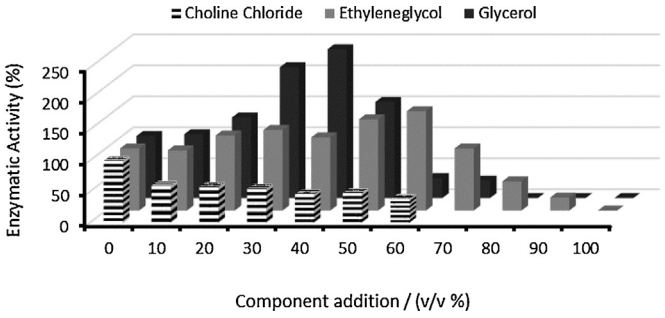

Fig. 2. Effect of polyols and choline chloride on the activity of α-rhamnosyl-β-glucosidase. One hundred percent activity corresponded to 1.55 ± 0.15 U/mL, 1.24 ± 0.19 U/mL and 3.93 ± 0.70 U/mL for Gly, EG and ChCl respectively.

donors (ethylene-glycol and glycerol) resulted in better co-solvents for the biocatalytic approach. More specifically, the enzyme displayed 95% activity at ChCl:Gly media with DES proportions of up to 40% (v/v).

Triggered by the observed results, studies using the DES components separately as co-solvents for the model reaction were conducted. When using DES as co-solvents in aqueous solutions, the existence of DES "clusters" – keeping its structure even at low concentrations –, has been recently suggested [25]. Thus, DES might keep some structural basis even at bulk aqueous solutions, being the actual (synergetic) responsible for the effect on biocatalysis, rather than their separate components [25]. Results when choline chloride, glycerol, and ethylene glycol were used as co-solvents are shown in Fig. 2. Choline chloride led to a deleterious effect for the glycosidase even at low additions (At proportions higher than 70% w/v choline chloride is not soluble in water and was thus not measured anymore). Remarkably, both polyols exhibited unexpected improvements of the enzymatic activity. Hesperetin production was raised up by 140% (3.74 ± 0.44 U/mL) at 40% v/v glycerol. Since transglycosylation of glycerol leading to rutinose-based glycerides could be an alternative explanation for the higher activity, the side

reaction would enable access to potentially interesting new compounds [28]. A pilot work in these conditions (40% v/v glycerol, a suspension of 1.8 mM hesperidin (1.1 g/L) and enzyme 1.55 U/mL, 1 h, 60 °C), led to a yield of 0.34 g hesperetin, accounting for 62% substrate conversion, in contrast with the 26% substrate conversion achieved when reactions were conducted in aqueous environments. Hence, the catalysis could also be favored by the stabilizing effect exerted by polyols [29]. Results suggest that an enhancement of hesperidin solubility would favor the catalysis up to the point where high viscosity of the media started to be adverse for the enzymatic process, presumably due to mass transfer limitations. Thus, the lower viscosity of the DESs in comparison with the single components – glycerol or ethylene-glycol – might be counteracted by the deleterious effect of the choline cation [26,30,31]. Quaternary ammonium cations are chaotropic ions (Hofmeister series); therefore, a negative effect can be expected on the enzyme activity [31]. Moreover, the interactions between choline chloride and hesperidin should not be neglected. Given that DES may be tailored according to actual (synthetic) necessities, it seems plausible to design novel polyol-based DES bearing more enzyme-compatible quaternary salts for glycosidases and flavonoid dissolution.

In summary, this communication has successfully explored the use of DES as co-solvents in flavonoid biocatalysis. Different choline chloride-based DES can be used for biocatalytic processes using wild-type glycosidases in practical frameworks of 30–40% DES (v/v). Given the industrial importance of flavonoids, and the prospective (environmental) advantages that the use of these co-solvents may have – when compared to more conventional and broadly used co-solvents such as DMSO – the use of DES open possibilities to combine biocatalysis with food industry, by using widely acceptable derivatives (such as glycerol). As lines for future research, the genetic design of robust biocatalysts that may be more active and stable at higher DES proportions may be a plausible option to increase substrate solubility. Furthermore, another approach would be to tailor novel DES with better structural properties for glycosidases, to broaden the positive effects of polyols and diminishing the deleterious influence caused by choline chloride.

Experimental

Production and purification of 6-*O*-α-rhamnosyl-β-glucosidase: The α-rhamnosyl-β-glucosidase was purified from the culture supernatant of *Acremonium* sp. DSM24697 as described before [6]. The procedure includes salt precipitation and hydrophobic interaction chromatography (Butyl-agarose) to yield 30.4% of the initial activity and purified 45.7 fold. The product was desalted and freeze-dried before storing at −18 °C.

Synthesis and characterization of DESs: Choline (CH):urea at molar ratio (1:2), with 40 rpm stirring blade at 90 °C for 1 h. CH: glycerol: molar ratio (1:2) without stirring at 70 °C for 30 min. CH:ethylene-glycol molar ratio (1:2) without agitation immersed 1 min at 80 °C.

Enzyme assay: For quantification of 6-O-α-rhamnosyl-β-glucosidase activity, each reaction contained 950 μL varying concentrations of the different DESs or DESs constituents, 1.8 mM hesperidin in 5 mM sodium phosphate buffer pH 6.0 and 50 μL enzyme solution and the reaction was carried out for 1 h at 60 °C. For transglycosylation reactions, 5–100% vol/vol DES, choline, glycerol and ethylene-glycol were added to the reaction mixture. The products of enzymatic reaction were analyzed by thin layer chromatography (Silicagel 60 W) using ethyl-acetate/2-propanol/water (3:2:2) as mobile phase and stained with anthrone reagent. The images were analyzed according to previous protocol reported by us [6]. The product (hesperetin) was quantified by HPLC using a KONIK-500-A series HPLC system attached to a KONIK UVIS 200 detector. The column was a reversed-phase Kromasil® Akzo-Nobel 100-5C18 (25 cm length, 4.6 mm internal diameter, pore size 100 Å, particle size: 5 μm). The elution consisted of an isocratic flow of 50% v/v methanol and 50% v/v water at a flow rate of 1.0 mL/min at 40 °C. Peak areas and extinction coefficient were calculated from chromatograms of authentic standards detected at 285 nm. Hesperetin extraction was performed twice placing samples (100 μL) with 400 μL of 50% v/v methanol and 50% v/v DMSO for 1 h at 50 °C. Then, samples were centrifuged (10,000 rpm, 10 min), the supernatants were suitably diluted in elution solvent. Triplicate samples were used. An enzyme unit was defined as the amount of enzyme required to produce 1 μmol of hesperetin per minute.

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