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

Gisela Weiz [a], Lucas Braun [a], Rosana Lopez [a], Pablo Domínguez de María [b], Javier D. Breccia [a,*]

[a] INCITAP—CONICET (National Scientific and Technical Research Council), Department of Chemistry, Faculty of Natural Sciences, National University of La Pampa (UNLPam), Av. Uruguay 151, 6300 Santa Rosa, La Pampa, Argentina
[b] Sustainable Momentum, SL. Ap. Correos 3517, 35004 Las Palmas de Gran Canaria, Canary Islands, Spain

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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.

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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-

* Corresponding author.
E-mail address: javierbreccia@gmail.com (J.D. Breccia).

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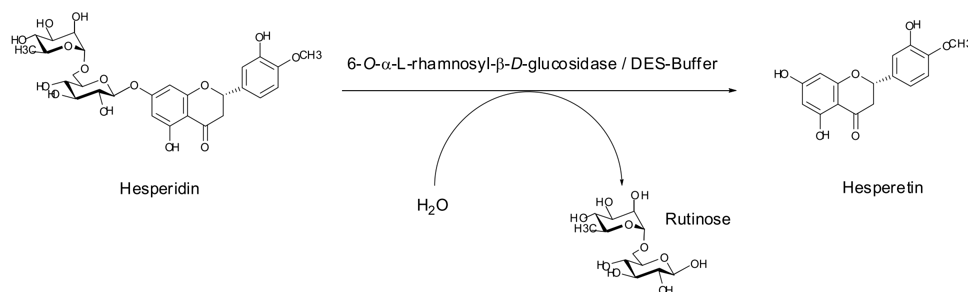

Scheme 1. One-step deglycosylation of hesperidin to afford hesperetin catalyzed by 6-*O*-α-rhamnosyl-β-glucosidase.

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

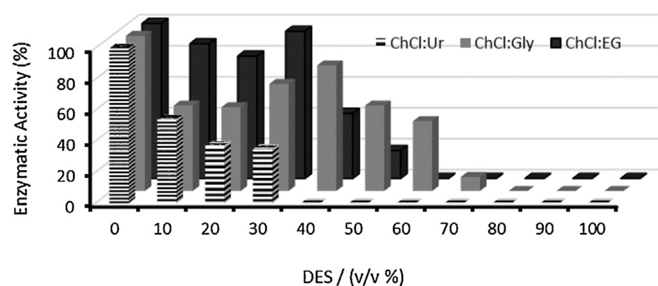

Fig. 1. Effect of DES concentration on the enzymatic activity for the deglycosylation of hesperidin by α-ramnosyl-β-glucosidase. See experimental part for reaction conditions. One hundred percent activity corresponded to 1.21 ± 0.17 U/mL, 1.30 ± 0.11 U/mL and 1.43 ± 0.10 U/mL for ChCl:EG, ChCl:Gly and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

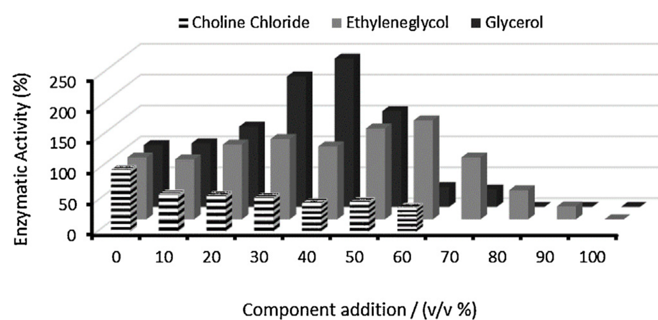

Fig. 2. Effect of polyols and choline chloride on the activity of α-rhamnosyl-β-glucosidase. One hundred percent activity corresponded to 1.55 ± 0.15 U/mL, 1.24 ± 0.19 U/mL and 3.93 ± 0.70 U/mL for Gly, EG and ChCl respectively.

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

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