Bull. Pharm. Sci., Assiut University, Vol. 28, Part 1, June 2005, pp. 113-117.

BIOTRANSFORMATION OF FLAVONOLS TO FLAVONOLS-3-O-GLUCOSIDE IN CELL CULTURES OF *ASTRAGALUS SIEBERI* DC.

A. M. A. Abd El-Mawla and Zedan Z. Ibraheim

Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, Assiut, Egypt

تم فى هذا البحث تنقية جزئية ووصف لانزيم جلوكوزيل ترانسفيريز من خلايا انسجة نبات استراجالاس سيبيراى دى سى التابع للعائلة القرنية. وقد تم الحصول على خلايا انسجة للنبات محل الدراسة بزراعة نبته لبذور معقمة للنبات فى وسط ام اس جامد مزود بـــ ٤،٥ ميكرومولار ١ نفثيل حامض الخليك و ٢،٣ ميكرومولار كاينيتين ثم تم نقل خلايا انسجة النبات الى وسط ام اس سائل مزود بنفس الهرمونات. وقد وجد ان الانزيم موضوع الدراسة أظهر أقصى فاعلية فى محلول منظم ذو أس هيدروجينى ٧،٥ ودرجة حرارة ٣٥ درجة مئوية ووجد ان المادة الأولية المفضلة للانزيم موضوع الدراسة كانت مادة الكامبيفيرول تليها مادة الكويرسيتين. وقد تم الكشف على نواتج الانزيم باستخدام طرق الكروماتوجرافيا المختلفة و تم التعرف عليهم باستخدام طرق التحاليل الطيفية المختلفة والمقارنة بعينات قياسية. تتيح هذه التجربة من الناحية الاقتصادية شروط جيدة لانتاج معدلات كبيرة من جلوكوزيدات الكامبيفيرول ، الكويرسيتين والازورامنيتين.

One distinct glucosyltransferase (GT) has been partially purified and characterized from cell cultures of Astragalus sieberi DC.; Family Leguminosae. Callus cultures were established from shoots of sterile germinated seeds maintained on solid MS medium supplemented with 4.5 μM 1-naphthylacetic acid (NAA) and 2.3 μM kinetin (KIN). The cell suspension cultures were obtained by transport of callus cultures to liquid MS medium with the same hormone supplementation. The GT was found to exhibit maximum activity at pH 7.5 and an incubation temperature of 35°. The preferred substrate of GT was found to be kaempferol, the second best substrate was quercetin. The isolated enzymatic products were detected by TLC and HPLC and identified by spectral analysis and comparison with authentic compounds.

This experiment from economic point of view provides the best conditions for large scale production of glucosides of kaempferol, quercetin and isorahmnetin.

INTRODUCTION

Three flavonoid glycosides, kaempferol-3-O-β-D-glucopyranoside (astragalin), kaempferol-3-O-rutinoside and kaempferol-3-O-(2-galactose-rhamnosyl-robinobioside) (mauritianin), as well as aglycones apigenin, kaempferol and quercetin, were isolated and identified from the cell suspension cultures of *Astragalus sieberi* DC.[1] These compounds exhibited hepatoprotective and free radical scavenging activities.[2-5]

Researches on the transfer of glucose to distinct flavonoid acceptor molecules are numerous,[6-12] yet only poor information on the enzymatic synthesis of flavonol-3-O-glucoside was found.[6,7] The commercial production of a pharmaceutical substance by plant cell culture has already been realized on an industrial scale in the case of shikonin, a naphthaquinone pigment with antibacterial, antiphlogistic and wound healing properties that is obtained from cell cultures of *Lithospermum erythrorhizon*,[13] for the same reason, our study deals with partial purification and characterization of the glucosyltransferase (GT), in addition, using this enzyme in large scale production of flavonols glucoside specially those compounds have reported biological activities as mentioned above.

MATERIALS AND METHODS

Chemicals

Kaempferol, quercetin, isorhamnetin, apigenin, luteolin, fisetin, galangin, myricetin, gossypetin, Kaempferol-3-O-glucoside, quercetin-3-O-glucoside and isorahmnetin-3-O-

Received in 19/12/2004 & Accepted in 22/3/2005

glucoside were obtained from Institute for Pharmaceutical Biology, University of Bonn, Germany.

UDP-D-glucose from Sigma, München. Dithiothreitol (DTT) from Applichem, Darmstadt. Polyclar AT from Serva, Heidelberg. Potassium dihydrogen phosphate, seasand and ammonium sulphate from Merck, Darmstadt.

Equipments

UV spectra were determined in Unicam SP-1750 ultraviolet spectrometer. EI-MS spectra were carried out on Hitachi M-80 and on MAT 311A, 70 ev. spectrometer. ^{1}H-NMR (500 MHz) spectra were determined in DMSO-d_6 using TMS as internal standard.

Cell cultures

Calli cultures of *Astragalus sieberi* DC. were established from the shoots of sterile germinated seeds maintained on solid MS (Murashige and Skoog),[14] containing 0.8% agar and 20 g/l sucrose and supplemented with 4.5 µM indole-3-acetic acid (IAA) and 2.3 µM kinetin (KIN). Calli cultures were initiated and maintained on solid MS medium at 25 ± 1° in the light. Cultures were sub-cultured at the end of exponential growth phase, at 4-week intervals.[1]

Cell-suspension cultures of *Astragalus sieberi* DC. were established as described previously[1] on liquid MS medium supplemented with 4.5 µM 1-naphthylacetic acid (NAA) and 2.3 µM kinetin (KIN) from calli grown on solid MS medium with the same hormone supplementation.

Buffer solution

The following buffer solution was used:
0.1 M potassium phosphate buffer (pH 7.5), containing 1.0 mM DTT.

Enzyme extraction

Cell-free extracts were prepared as described in literatures.[15,16] Cells (4 g) were mixed with 0.4 g polyclar AT and a spatula of seasand and homogenised for 15 min in 2.5 ml 0.1 M potassium phosphate buffer (pH 7.5) containing 1.0 mM DTT. After centrifugation in a super-speed refrigerated centrifuge at 12000 r.p.m. and 4° for 20 min the supernatant was passed through a PD_{10} column (Amersham Pharmacia Biotech) and the high-molecular weight fraction was eluted with 3.5 ml of the same buffer.

Enzyme assay

The assay system[17] contained in 100 µl total volume: 65 µl 0.1 M potassium phosphate buffer (pH 7.5), 30 µl enzyme extract (about 15-25 µg protein), 700 nmol uridine 5`-diphosphoglucose (UDPGlc) and 50 nmol kaempferol. The reaction was started by addition of UDPGlc. The incubation was carried out for 15 min at 35° and was terminated by addition of 40 µl chloroform-methanol, (2:1, v/v; plus 0.5% HCl), resulting in a Folch partition.[18] The flavonoids were concentrated in the upper phase and the reaction mixture was analysed by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC).[1]

A control experiment was carried out by further incubation as mentioned above, using denatured protein (boiled enzyme extract); 100 µl enzyme extract was heated in boiling water bath for 10 min (heat inactivation) and from which 30 µl was added to the incubation assay.

Protein determination

Protein was determined as described by Bradford.[19]

Ammonium sulphate precipitation

A cell-free extract (25 ml) obtained from 40 g of freshly harvested cells was fractionated by addition of ammonium sulphate, reaching 70% saturation in 10% steps.[20]

Determination of the pH and temperature optima

The assay for the enzyme studied was carried out at different pH values between 6.0 and 9.0. At the optimum pH value another series of incubations were performed at different temperatures between 20 and 55°. Two independent experiments were performed and mean values were calculated.

Study of substrate specificity

At the pH and temperature optima, enzyme assay was performed using a series of related substrates for the enzyme studied. All substrate concentrations were saturating, i.e. a further increase in substrate concentration did

not lead to a further increase in product amount.

Analytical methods

Analysis of the enzymatic product was performed by HPLC and TLC. Fifty µl of the upper phase were injected into an HPLC apparatus (L-6200 A intelligent pump, L-4000 UV detector, Merck Hitachi, Japan) equipped with a Nucleosile 100-5 C_{18} column (Macherey-Nagel, Düren, Germany). Eluents were water (A) and methanol (B), both containing 1% phosphoric acid. The flow rate was 1 ml/min. Flavonoids were eluted with a linear gradient: 20% B for 4 min, 20 to 70% B for 22 min, 70-100% B for 2 min. Detection was done at a wavelength of 366 nm. The retention time (R_t) for the enzymatic product was 10 min and for its aglycone precursor (kaempferol) was 24 min.

The enzymatic product was isolated by preparative TLC on silica gel 60 F_{254}-coated aluminium sheets (Merck, Darmstadt) using $CHCl_3$-CH_3OH (7: 3) as a solvent system, further purification was carried on HPLC.[1] The R_F value for the enzymatic product was 0.41 and it was 0.83 for its aglycone kaempferol.

RESULTS AND DISCUSSION

Results

Incubation of Kaempferol and UDPGlc with desalted cell-free extract from *Astragalus sieberi* cell cultures resulted in the formation of kaempferol-3-O-β-D-glucopyranoside (Fig. 1). The identity of the enzymatic product was shown by co-chromatography (TLC and HPLC) with a sample of authentic compound and by comparing its spectroscopic data (UV, MS, ^{1}H-NMR) with published data.[1,21] UV: λ_{max} (CH_3OH, nm) 266, 371; λ_{max} (CH_3OH + $NaOCH_3$, nm) 278, 416; λ_{max} (CH_3OH + NaOAc, nm) 276, 393; λ_{max} (CH_3OH + $NaOAc/H_3BO_3$, nm) 269, 373; λ_{max} (CH_3OH + $AlCl_3$, nm) 272, 375; λ_{max} (CH_3OH + $AlCl_3$/ HCl, nm) 272, 373. EI-MS *m/z* (rel. int.): 286 (16) [M^+ agl.], 258 (11), 153 (4), 149 (33), 125 (8), 61 (100), 43 (34). ^{1}H-NMR (500 MHz, DMSO-d_6): δ 5.50 (1 H, d, J= 7.1 Hz, H-1 glu), 6.21 (1H, d, J= 2.1 Hz, H-6), 6.46 (1H, d, J= 2.1 Hz, H-8), 7.10 (2H, dd, J= 8.4, 1.4 Hz, H-`3, H-`5), 8.10 (2H, dd, J= 8.4, 1.4 Hz, H-`2, H-`6).

No product formation occurred when protein extracts were heat inactivated. Protein extracts were fractionated by stepwise addition of ammonium sulphate (Fig. 2). The protein fraction which precipitated between 50 and 60% ammonium sulphate saturation contained high glucosyltransferase activity. The enzyme exhibited maximum activity at pH 7.5 and 35° (Fig. 3).

Studies of the substrate specificity of glucosyltransferase revealed that the preferred substrate was really kaempferol (Table 1). The second best substrate was quercetin and the third one was isorhamnetin.

The other enzymatic products; quercetin-3-O-glucoside and isorhamnetin-3-O-glucoside were identified by co-chromatography (TLC and HPLC) with a samples of authentic compounds, in addition comparing their UV and melting points with reported data.[2-5, 21]

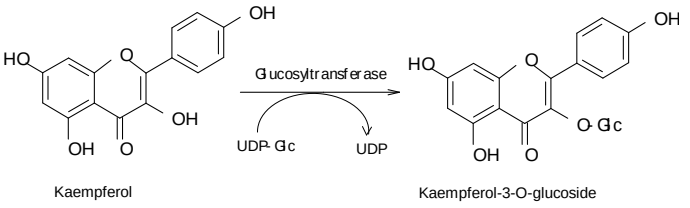

Fig. 1: Reaction catalysed by glucosyltransferase

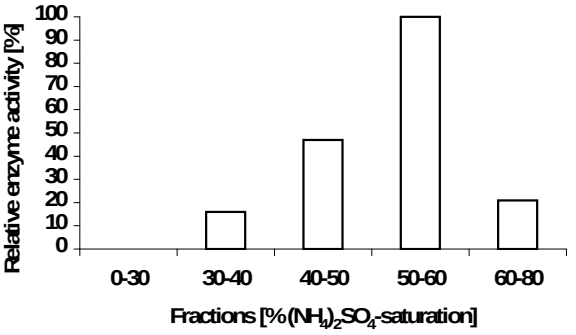

Fig. 2: Distribution of UDP-glucose: flavonol 3-O-glucosyltransferase activity in ammonium sulphate-precipitated protein fractions from *Astragalus sieberi* cell cultures

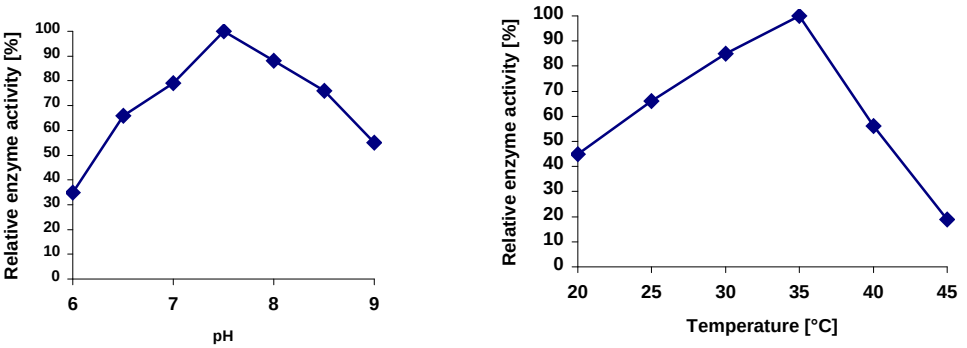

Fig. 3: pH and temperature optima of UDP-glucose: flavonol 3-O-glucosyltransferase from *Astragalus sieberi* cell cultures

Table 1: Substrate specificity of UDP-glucose: flavonol 3-O-glucosyltransferase from *Astragalus sieberi* cell cultures. Cell free extracts were subjected to ammonium sulphate precipitation. The 50-60% fraction (Fig. 1) was used to study the substrate specificity.

Substrate	Relative enzyme activity (%)
Kaempferol	100
Quercetin	48
Isorhamnetin	18
Apigenin	0
Luteolin	0
Fisetin	0
Galangin	0
Myricetin	0
Gossypetin	0

Discussion

In cell suspension cultures of *Astragalus sieberi* DC., a distinct glucosyltransferase (GT) has been partially purified and characterized. It catalyzes the transfer of the glucosyl moiety of uridine 5`-diphosphoglucose (UDPGlc) to the 3-hydroxyl group of flavonol aglycones.

The investigation reported here was closely related to others performed on different systems such as intact plants and cell cultures[6-9] Moreover, the UDP-glucose: flavonol 3-O-glucosyltransferase has also been isolated from cell suspension cultures of parsley[8] and from anthers of *Tulipa* cv. Apeldoorn.[17] Most properties of the investigated enzyme are in accordance with those reported from the literature: pH and temperature optima are 7.5 and 35°, respectively.[9,12] Cations like Mg^{2+}, NH_4 and Ca^{2+} as well as EDTA did not or only poorly affect GT which agree with results reported.[7,23,24] With regards to its substrate specificity (Table 1), GT from *Astragalus sieberi* cell cultures predominantly glucosylates flavonols particularly flavonol aglycones like kaempferol, quercetin and isorhamnetin. Activity could not be detected with flavones like apigenin and luteolin. The enzyme acts specifically in the 3-O-position; the 7-O-glucosyltransferase activity previously described for enzyme preparations from parsley cell suspension cultures[7,8,11] could not be detected.

From the previous work, we came to a conclusion that; the application of protein extract of *Astragalus sieberi* proved to be successful for the large scale production of glucosides of kaempferol, quercetin and isorahmnetin in the laboratory. In addition, this work presents in the future a good start for carrying out genetic study of glucosyl-transferase (GT). Genetic engineering as allowed the production of plants with an altered content of secondary metabolites, gene expression of GT in *Astragalus sieberi* cell cultures will lead to strong increase of kaempferol-3-O-β-D-glucopyranoside and carrying out such experiments in the future may prove useful for the production of secondary metabolites of pharmaceutical

importance, both by intact plants and by plant cell cultures.

Accordingly, we recommend the application of this technique in industry aiming for producing those types of glucosides in large amount enough for market requirements.

Acknowledgement

We would like to thank Dr. G. Eckhardt and Dr. W. Tomberg (Institute for Organic Chemistry, University of Bonn, Germany) for performing mass and ^{1}H-NMR spectra, respectively.

REFERENCES

1- A. M. A. Abd El-Mawla and A. A. Attia, Bull. Pharm. Sci., Assiut University, 25: 79-83 (2002).
2- C. W. Beninger and G. L. Hosfield, J. Agric. Food Chem., 27, 7879-7883 (2003).
3- L. Parejo, F. Viladomat, J. Bastida, G. Schmeda-Hirschmann, J. Burillo and C. Codina, J. Agric. Food Chem., 7, 1890-1897 (2004).
4- K. M. Janisch, G. Williamson, P. Needs and G. W. Plumb, Free Radic. Res., 8, 877-884 (2004).
5- H. Oh, D. H. Kim, J. H. Cho and Y. C. Kim, J. Ethnopharmacol, 2-3, 421-424 (2004).
6- G. A. Barber, Biochemistry, 1, 463-468 (1962).
7- A.Sutter, R. Ortmann and H. Grisebach, Biochem. Biophys. Acta, 258, 71-87 (1972).
8- A. Sutter and H. Grisebach, Biochem. Biophys. Acta, 309, 289-295 (1973).
9- J. E. Poulten and M. Kauer, Planta, 136, 53-59 (1977).
10- K. F. F: Kho, J. Kamsteeg and J. van Brederode, Z. Pflanzenphysiol., 88, 449-464 (1978).
11- J. Köster and W. Barz, Arch. Biochem. Biophys., 212, 98-104 (1981).
12- W. Hösel, Glucosylation and glucosidases. In: The Biochemistry of plants, (E. E. Conn, ed.), vol. 7, Secondary Plant Products, pp. 725-753 (1981), Academic Press, New York, London, Toronto, Sydney, San Francisco.
13- M. Tabata and Y. Fujita, Production of shikonin by plant cell cultures. In P Day, M, Zaitlin, A, ollaender, eds., Biotechnology in Plant Science, Academic Press, Orlando, FL, pp. 207-218 (1985).
14- T. Murashige and F. Skoog, Physiol. Plant., 15, 473 (1962).
15- S. Peters, W. Schmidt and L. Beerhues, Planta, 204, 64-69 (1998).
16- A. M. A. Abd El-Mawla, W. Schmidt and L. Beerhues, Planta, 212, 288-293 (2001).
17- M. Teusch, G. Forkmann and W. Seyfferet, Planta, 168, 586-591 (1986).
18- J. Folch, M. Less and G. H. Saane-Stanley, J. Biol. Chem., 226, 496-509 (1957).
19- M. M. Bradford, Analyt. Biochem., 72, 248-254 (1976).
20- W. Barillas and L. Beerhues, Planta, 202, 112-116 (1997).
21- J. Harborne and T. Mabry, ``The flavonoids: Advances in Research``, 1st Ed., University Press, Cambridge, pp. 98-99 (1982).
22- G. Kleinehollenhorst, H. Behrens, G. Pegels, N. Srunk and R. Wiermann, Z. Naturforsch., 37c, 587-599 (1982)
23- R. K. Ibrahim and H. Griesbach, Arch. Biochem. Biophys., 176, 700-708 (1976).
24- R. K., Ibrahim, Z. Pflanzenphysiol., 85, 253-262 (1977).