FEBS Letters 468 (2000) 166–170 FEBS 23355

Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase

Andrea J. Day[a,*], F. Javier Cañada[b], Juan C. Díaz[b], Paul A. Kroon[a], Russell Mclauchlan[a], Craig B. Faulds[a], Geoff W. Plumb[a], Michael R.A. Morgan[c], Gary Williamson[a]

[a] *Diet, Health and Consumer Science Division, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK*
[b] *Instituto de Química Orgánica, Consejo Superior de Investigaciones Científicas, Juan de la Cierva 3, 28006 Madrid, Spain*
[c] *Proctor Department of Food Science, University of Leeds, Leeds LS2 9JT, UK*

Received 21 January 2000

Edited by Barry Halliwell

Abstract Lactase phlorizin hydrolase (LPH; EC 3.2.1.62) is a membrane-bound, family 1 β-glycosidase found on the brush border of the mammalian small intestine. LPH, purified from sheep small intestine, was capable of hydrolysing a range of flavonol and isoflavone glycosides. The catalytic efficiency (k_{cat}/K_m) for the hydrolysis of quercetin-4′-glucoside, quercetin-3-glucoside, genistein-7-glucoside and daidzein-7-glucoside was 170, 137, 77 and 14 ($mM^{-1}\ s^{-1}$) respectively. The majority of the activity occurred at the lactase and not phlorizin hydrolase site. The ability of LPH to deglycosylate dietary (iso)flavonoid glycosides suggests a possible role for this enzyme in the metabolism of these biologically active compounds.

© 2000 Federation of European Biochemical Societies.

Key words: Flavonol glycoside; Isoflavone; Phytoestrogen; Lactase phlorizin hydrolase; β-Glucosidase; Absorption

1. Introduction

Large quantities of potentially beneficial or toxic plant phenolics are present in human diets. Although the range of plant phenolics is diverse, the majority are glycosylated, and this affects uptake, metabolism and subsequent biological activity. Flavonoids, a major class of plant phenolics found widely in fruits and vegetables, tea and red wine [1], may reduce the risk of heart disease and stroke [2]. Isoflavones are phytoestrogens which have potent biological activity that may reduce the risk of hormone-dependent disease [3]. For example, isoflavones from soya significantly lengthened the follicular phase of the menstrual cycle [4], an action which has been associated with a decreased risk of breast cancer. Glycosylation of the (iso)-flavonoids had been thought to delay intestinal absorption until the large intestine where metabolism by colonic microflora releases aglycones [1]. However, metabolites of some flavonols and isoflavones appear in plasma within 30 min of ingestion, indicating rapid absorption in the small intestine [5,6].

Various mammalian β-glucosidases are present in the small intestine. These include, glucocerebrosidase (EC 3.2.1.62) [7], lactase phlorizin hydrolase (LPH) (EC 3.2.1.23 and 62) [8], broad-specificity cytosolic β-glucosidase (EC 3.2.1.21) [7] and pyridoxine glucoside hydrolase [9]. All of these enzymes, with the exception of LPH, act intracellularly and would require transport of the intact (iso)flavonoid glycoside if they were to play a role in the metabolism of these compounds. LPH, however, is present on the luminal side of the brush border in the small intestine and can act on dietary glycosides before absorption. LPH is primarily responsible for the hydrolysis of lactose from milk during infant years [10]. The enzyme is genetically regulated with levels normally declining during adolescence. Over 75% of the world's population have the non-persistent phenotype and will have low lactase levels as adults resulting in lactose maldigestion. However, populations with a history of consuming dairy produce, such as the Northern Europeans, have persistence of LPH into adulthood [11]. LPH has a second active site, both identified on the same protein [12,13], capable of hydrolysing another component of milk, β-glycosylceramide, and the dihydrochalcone glucoside, phlorizin [14].

In a previous study we showed that cell-free extracts of human small intestine and liver had β-glucosidase activity towards flavonoid and isoflavone glycosides [15]. The activity was due to a broad-specificity, cytosolic β-glucosidase recently confirmed by Lambert et al. [16]. The liver β-glucosidase was capable of hydrolysing various (iso)flavonoid glucosides, although of the flavonols only quercetin-4′-glucoside was hydrolysed, not quercetin-3-glucoside or quercetin-3,4′-diglucoside. A similar pattern of hydrolysis was observed with the cell-free extracts of human small intestine, although additional hydrolysis of quercetin-3-glucoside was also observed. Deglycosylation of quercetin-3-glucoside in the small intestine may result from activity of a second β-glucosidase such as LPH. As the (iso)flavonoid glucosides are very similar in structure to the dihydrochalcone glucosides (Fig. 1), we aimed to test the hypothesis that these compounds may be substrates for LPH and determine which domain of LPH was responsible for any associated activity.

2. Materials and methods

2.1. Chemicals

LPH was purified from lamb small intestine to one major band on SDS–PAGE as previously described [17]. All (iso)flavonoids were purchased from Extrasynthese (Genay, France), except phlorizin and phloretin which were purchased from Sigma (Poole, UK) and quercetin-3,4′-diglucoside which was extracted and purified (>99%) from onion bulb tissue [18]. Glucal and 2′,4′-dinitrophenyl-2-fluoro-2-deoxy-β-D-glucopyranoside (2F-DNPglc) were purchased from Sigma. Glucose diagnostic kit (Trinder) and all other reagents were purchased from Sigma and were of analytical reagent grade or HPLC grade

*Corresponding author. Fax: (44)-1603-507723.
E-mail: andrea.day@bbsrc.ac.uk

0014-5793/00/$20.00 © 2000 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(00)01211-4

where applicable. Water was purified via a Millex Q-plus system (Millipore, Watford, UK).

2.2. Preparation of flavonoids

All substrates were dissolved in dimethylsulphoxide (DMSO) and were stable in this form when stored at 4°C. Immediately prior to the reaction, an aliquot was diluted in sodium phosphate buffer (50 mM, pH 6) to give a final DMSO concentration of less than 0.1% (v/v) in the reaction mixture. Genistein-7-glucoside diluted in buffer was heated to 37°C during preparation to avoid precipitation.

2.3. Enzyme assay

Initial incubations to determine substrate specificity of LPH were carried out with each (iso)flavonoid glycoside: quercetin-4′-glucoside, quercetin-3-glucoside, quercetin-3,4′-diglucoside, 3′-methylquercetin-3-glucoside, genistein-7-glucoside and daidzein-7-glucoside, quercetin-3-rhamnoglucoside and naringenin-7-rhamnoglucoside (100 μM), incubated with LPH (final concentration 1 μg/ml) and phosphate buffer to a volume of 0.1 ml (20 min, 37°C). The reaction was stopped with 0.15 ml acetonitrile–water (30/70 v/v), followed by centrifugation (13 600 × *g*, 10 min, 4°C). Recovery of the (iso)flavonoid glycoside was greater than 99%. The supernatant was filtered through 0.22 μm PTFE filter units (HPLC Technology Company, Macclesfield, UK) and analysed by HPLC. To determine the K_m and V_{max}, various concentrations of quercetin-4′-glucoside, quercetin-3-glucoside, genistein-7-glucoside and daidzein-7-glucoside were mixed with LPH (final concentration 0.1 μg/ml) and phosphate buffer to a volume of 0.2 ml. All samples were incubated at 37°C for a time period during which the reaction was linear (5–30 min depending on substrate). The reaction was stopped with acetonitrile–water (0.3 ml) as before and the sample treated for HPLC analysis as above. The amount of substrate converted was calculated from standard curves of peak areas produced by injecting known amounts of various (iso)flavonoids directly onto the column. K_m and V_{max} were calculated using a method described by Wilkinson [19].

2.4. Inactivation of the phlorizin hydrolase active site of LPH

LPH was preincubated in the presence of a high concentration of glucal (30 mM, to protect the lactase site from inactivation), for 5 min before the addition of 2F-DNPglc (0.15 mM). The mixture was incubated for 10 min and the glucal and excess inhibitor were removed by ultrafiltration (Centricon P-10) before measuring the residual activity of the substrates: lactose (100 mM), phlorizin (1 mM), quercetin-3-glucoside (1 mM) and quercetin-4′-glucoside (1 mM). Lactose hydrolysis was measured using coupled assays with glucose oxidase (Trinder reagent). Phlorizin and flavonol glycoside hydrolysis were determined by HPLC as the aglycone released.

2.5. HPLC analysis of flavonoid glycoside hydrolysis

A modified version of the analytical HPLC method of Price et al. [20] was used. Solvents A (water–tetrahydrofuran–trifluoroacetic acid 98:2:0.1 v/v) and B (acetonitrile) were run at a flow rate of 1 ml/min, using a gradient of 17% B (2 min), increasing to 25% B (5 min), 35% B (8 min), 50% B (5 min) and then to 100% B (5 min). A column clean-up stage maintained B at 100% (5 min) followed by a re-equilibration at 17% B (15 min). The column was packed with Prodigy 5 μm ODS3 reverse-phase silica, 250 mm by 4.6 mm i.d. (Phenomenex, Macclesfield, UK). Diode array detection monitored the eluant at 270 and 370 nm. The appropriate (iso)flavonoid glycoside for each enzyme kinetic analysis was used as an external standard at concentration ranging from 0 to 250 μM (400 μM for daidzein-7-glucoside). The (iso)flavonoid glycosides were separated from their respective aglycones under the above HPLC conditions by between 6 and 10 min. The product in each of the reactions was confirmed by co-elution of peak with standard compounds and by matching UV spectra. Identity of original compounds was confirmed by mass spectrometry and nuclear magnetic resonance.

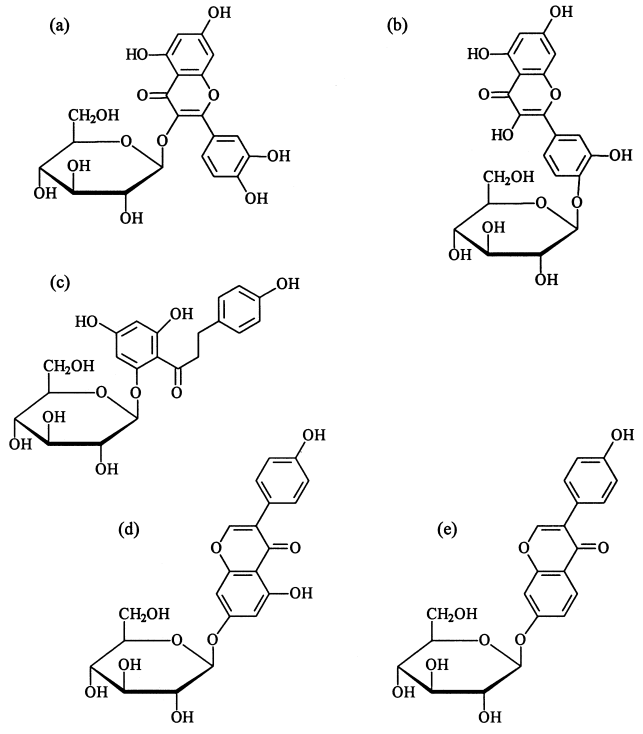

Fig. 1. Structure of (a) quercetin-3-glucoside, (b) quercetin-4′-glucoside, (c) phlorizin, (d) genistein-7-glucoside and (e) daidzein-7-glucoside.

3. Results

LPH deglycosylated the following substrates: quercetin-4′-glucoside, quercetin-3-glucoside, quercetin-3,4′-diglucoside, 3′-methylquercetin-3-glucoside, genistein-7-glucoside and daidzein-7-glucoside. However, LPH did not hydrolyse quercetin-3-rhamnoglucoside (rutin) or naringenin-7-rhamnoglucoside (naringin); both are common flavonoid glycosides in the diet. Fig. 2 shows representative chromatograms from 0 and 20 min incubations of quercetin-3-glucoside in the presence of LPH. The K_m and V_{max} for the deglycosylation of various isoflavone and flavonol glycosides are shown in Table 1. The catalytic efficiency (k_{cat}/K_m) suggests that the quercetin glucosides are hydrolysed more effectively than the isoflavone glucosides.

Table 1
Kinetic constants for the hydrolysis of flavonol and isoflavone glucosides by LPH purified from lamb small intestine [17]

	K_m (μM)	V_{max} (U mg^{-1} LPH)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Quercetin-4′-glucoside	44 ± 7	3.2 ± 0.18	7.5	170
Quercetin-3-glucoside	46 ± 6	2.7 ± 0.13	6.3	137
Genistein-7-glucoside	85 ± 11	2.8 ± 0.18	6.5	77
Daidzein-7-glucoside	149 ± 18	0.9 ± 0.04	2.1	14
Lactose	8800 ± 300[a]	14.5 ± 0.5[a]	33.8	4
Phlorizin	2 ± 0.1[a]	0.22 ± 0.002[a]	0.5	257

One unit of activity is defined as the amount of enzyme releasing 1 μmol of product per min at 37°C, pH 6.
[a]Values taken from [17]

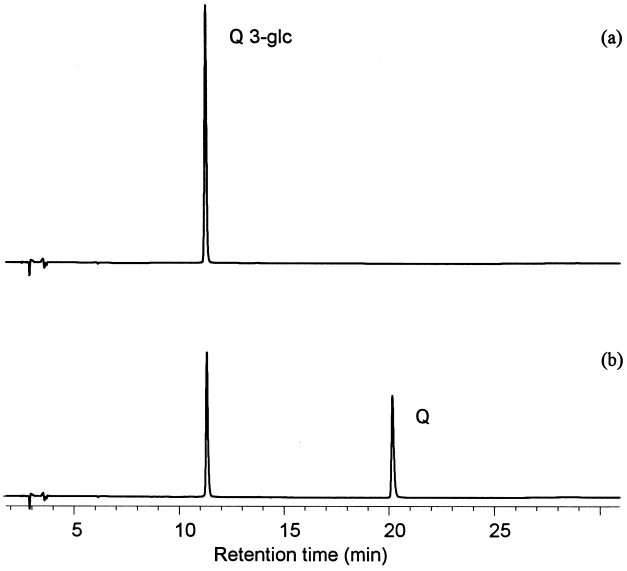

Fig. 2. HPLC chromatogram monitoring relative absorption at 370 nm of quercetin-3-glucoside (Q3-glc) and quercetin (Q) after incubation with lactase phlorizin hydrolase at 37°C for (a) 0 min, (b) 20 min.

To determine which site of LPH was responsible for the activity on the quercetin glucosides, a mechanism-based inhibitor of glycosidase, 2F-DNPglc [21], was incubated with the enzyme. 2F-DNPglc rapidly inhibits phlorizin hydrolase by covalently binding to the glutamic acid residues in the active site [22]. Lactase activity is also inhibited, but the reaction occurs more slowly. Pre-incubation with glucal protects the lactase active site from inactivation allowing the individual contributions of each active site to be calculated [22]. Fig. 3 shows the residual activity of LPH after specific inactivation of the phlorizin hydrolase site by 2F-DNPglc. Lactose was hydrolysed only at the lactase active site, as shown by complete activity after incubation of LPH with 2F-DNPglc following protection with glucal. LPH retained only 14% of activity towards phlorizin, confirming the major active site for this compound is the phlorizin hydrolase site, with the lactase site contributing only a small percentage of activity as expected. Conversely, LPH retained 85% and 70% of activity

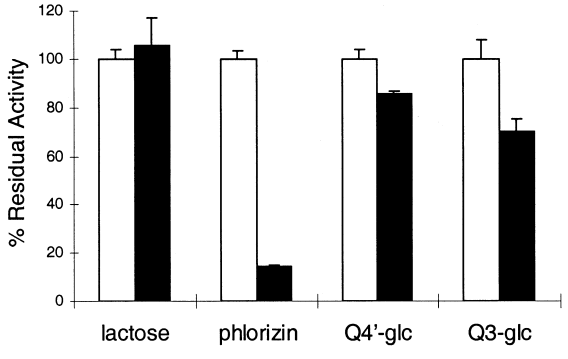

Fig. 3. Specific inactivation of the phlorizin hydrolase activity. Activity is expressed relative to unmodified LPH for lactose, phlorizin, quercetin-4′-glucoside (Q4′-glc) and quercetin-3-glucoside (Q3-glc). White bars: preincubation of LPH without 2F-DNPglc; filled bars: preincubation of LPH with 2F-DNPglc (0.15 mM, 10 min). Error bars represent the standard deviation for at least three replicates.

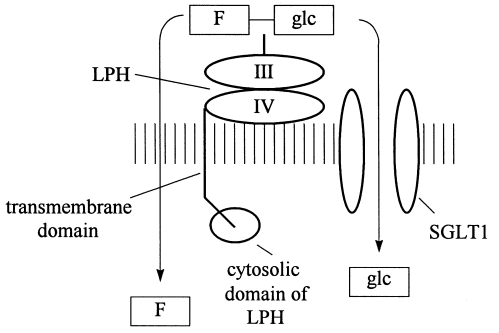

Fig. 4. Potential mechanism for absorption of flavonoid glucosides in the mammalian small intestine. Domains III and IV of LPH are shown as a transmembrane protein with diffusion of released aglycone and glucosides (F, flavonoid; glc, glucose). SGLT1, the sodium-dependent glucose transporter, will rapidly transport the glucose released from the action of LPH, and may be capable of transporting some smaller phenolic glycosides.

on quercetin-4′-glucoside and quercetin-3-glucoside respectively, suggesting that the major contribution to the hydrolysis of these compounds is from the lactase site. The phlorizin hydrolase site is responsible for the remaining smaller proportion of the activity.

4. Discussion

Certain flavonoid glycosides are absorbed from the small intestine in man. Specifically, quercetin was detected in plasma at peak levels within 20 min of volunteers ingesting onions [5]. Genistein and daidzein were also detected in the plasma of volunteers 30 min after a soya meal, although highest plasma levels correspond to subsequent absorption in the colon [6]. Diffusion of hydrophilic (iso)flavonoid glycosides across biological membranes is unlikely to occur, and so the absorption demonstrated requires either hydrolysis of the β-glucoside or a specific active transport mechanism. Although the small intestine does support a relatively small population of bacteria, microbial hydrolysis is unlikely to account for the observed levels of early absorption of these compounds in humans. Neither the acidic conditions of the stomach nor the digestive enzymes secreted by the stomach or pancreas hydrolyse β-glucosides [23]. The only mammalian β-glucosidase to have an activity within the gut lumen is LPH, but the ability of this enzyme to deglycosylate (iso)flavonoid glycosides has not previously been investigated.

The present study demonstrates that LPH is active on various (iso)flavonoid glucosides with a high affinity towards the flavonol glucosides in particular. Although sheep LPH was used the enzyme has been shown to have a comparable activity towards lactose as other sources of LPH, such as human, rat, bovine, monkey and pig [17], and is thus a good representative of this class of β-glycosidase. All of these mammalian LPHs have two catalytic sites [10], one to hydrolyse lactose and the other involved in the deglycosylation of more hydrophobic substrates, such as phlorizin [14]. Although the (iso)flavonoid glycosides are structurally related to phlorizin, specific inactivation of the phlorizin hydrolase site by 2F-DNPglc clearly indicated that the lactase site was mainly responsible for the hydrolysis of the flavonol glucosides. Lactase has a preference for hydrophilic substrates, but will accept a

broad range of aglucones. An available hydroxyl in the correct orientation to the active site for hydrogen bonding appears to be the major requirement [24]. Although phlorizin hydrolase accepts more hydrophobic glycosides, specificity of the active site may result from orientation of the glucose residue with the aglycone.

LPH is present in the small intestine of all infant mammals and is primarily responsible for the hydrolysis of lactose from milk prior to weaning. Humans are unique to other mammals in this respect, as high levels of LPH can persist into adulthood. LPH is present in the brush border of the small intestine, and therefore hydrolysis of the flavonoid glycosides will occur within the gut lumen. The more hydrophobic aglycone released can then diffuse into the epithelial cells. Hanke et al. [25] provided evidence for the preferential uptake of glucose released from phlorizin by LPH compared to free glucose in solution. The rapid transfer into the enterocyte of the released glucose is due to an efficient transport mechanism in parallel to the β-glucosidase activity. In the intestine, LPH protrudes into an unstirred boundary layer and is positioned in close proximity to the sodium-dependent glucose transporter, SGLT1. The aglycone released by the action of LPH may result in an increased local concentration, stimulating diffusion across the brush border. Rapid conjugation within the enterocyte to glucuronide and sulfate, and blood flow to remove the conjugates, will maintain a concentration gradient of the aglycone with the gut lumen. Fig. 4 shows the potential mechanism of flavonoid glycoside absorption by LPH.

Transport of flavonol glycosides by SGLT1 has been suggested [26] and results from experiments using rat everted sacs have established that quercetin glucosides can interact with SGLT1 [18]. This does not necessarily indicate that transport occurs, as phenolic glucosides could interact by inhibiting sugar transport but not themselves be transported across the membrane. For example, phlorizin is an inhibitor of SGLT1 but is not transported across the brush border due to the bulky nature of the aglycone [27]. As the flavonoid glucosides are similar to phlorizin in size, it would seem unlikely that they would be transported by SGLT1. Recent work by Spencer et al. [28] found a substantial proportion of quercetin-3-glucoside absorbed intact after a 90 min incubation of rat perfused small intestine. Hydrolysis of quercetin-3-glucoside and other flavonoid glucosides had also occurred during absorption, as evident by the presence of the aglycone and glucuronide metabolites. In contrast however, Crespy et al. [29] and work carried out in our laboratory (unpublished) have not found any evidence of intact quercetin-3-glucoside after incubation for a shorter time (15–20 min) with in situ perfused rat intestine or everted jejunum, although quercetin glucuronides were formed. Despite these apparent differences partial or full hydrolysis of flavonoid glucosides occurs during absorption. This indicates β-glucosidase activity arising from the action of LPH or broad-specificity cytosolic β-glucosidase after transport of the intact glucoside by SGLT1.

A major implication of the hypothesis that LPH is involved in the uptake of flavonoid glycosides is a reduced level of absorption in the small intestine of the population expressing the non-persistent phenotype for LPH. If absorption does not occur in the small intestine, the flavonoids will be subjected to microbial metabolism and extensive degradation in the colon. Conversely, isoflavones appear to be more resistant to microbial degradation, but are metabolised by the microflora to more oestrogenic compounds [30]. Thus a reduced level of isoflavone glycoside absorption in the small intestine could result in an increased absorption of more biologically active compounds formed in the colon. It is interesting to note that other mammals, such as rat and sheep, have considerably higher microbial populations in the stomach and hence hydrolysis of the glycosidic link by LPH will be of little importance in the uptake of (iso)flavonoids.

In conclusion, LPH is capable of hydrolysing various flavonol and isoflavone glucosides. This enzymic action allows us to postulate a mechanism for absorption in the small intestine of these biologically active compounds. Surprisingly, the majority of the activity is from the lactase domain of LPH with only a small contribution from the phlorizin hydrolase domain. The presence of LPH activity in the gut lumen would indicate a role in the metabolism of (iso)flavonoid glycosides from the diet, but it is necessary to assess the potential role of LPH on uptake of phenolic glycosides in humans.

Acknowledgements: We would like to thank the Biotechnology and Biological Sciences Research Council for funding and for a Ph.D. studentship (to A.J.D.) and the Spanish Ministry of Education for a Fellowship (to J.C.D.) and project funding (PB96-0833, to F.J.C.).

References

[1] Kühnau, J. (1976) World Rev. Nutr. Diet 24, 117–191.
[2] Middleton, E. and Kandaswami, C. (1994) in: The Flavonoids: Advances in Research since 1986 (Harborne, J.B., Ed.), pp. 619–652, Chapman and Hall, London.
[3] Adlercreutz, H. (1995) Environ. Health Perspect. 103 (Suppl. 7), 103–112.
[4] Cassidy, A., Bingham, S. and Setchell, K.D. (1994) Am. J. Clin. Nutr. 60, 333–340.
[5] Hollman, P.C.H., Van Trijp, J.H., Buysman, M.N.C.P., Gaag, M.S., Menglers, M.J.B., de Vries, J.H.M. and Katan, M.B. (1997) FEBS Lett. 418, 152–156.
[6] King, R. and Bursill, D.B. (1998) Am. J. Clin. Nutr. 67, 867–872.
[7] Daniels, L.B., Coyle, P.J., Chiao, Y. and Glew, R.H. (1981) J. Biol. Chem. 256, 13004–13013.
[8] Dahlqvist, A. and Borgström, B. (1961) Biochem. J. 81, 411–418.
[9] McMahon, L.G., Nakano, H., Levy, M.-D. and Gregory III, J.F. (1997) J. Biol. Chem. 272, 32025–32033.
[10] Semenza, G. (1987) in: Mammalian Ectoenzymes (Kenny, A.J. and Turner, A.J., Eds.), pp. 256–287, Elsevier, Amsterdam.
[11] Flatz, G. (1987) in: Advances in Human Genetics (Harris, H. and Hirschhorn, K., Eds.), pp. 1–77, Plenum Press, New York.
[12] Wacker, W., Keller, P., Falchetto, R., Legler, G. and Semenza, G. (1992) J. Biol. Chem. 267, 18744–18752.
[13] Zecca, L., Mesonero, J.E., Stutz, A., Poiree, J.C., Giudiceelli, J., Cursio, R., Gloor, S.M. and Semenza, G. (1998) FEBS Lett. 435, 225–228.
[14] Leese, H.J. and Semenza, G. (1973) J. Biol. Chem. 248, 8170–8173.
[15] Day, A.J., DuPont, M.S., Ridley, S., Rhodes, M., Rhodes, M.J.C., Morgan, M.R.A. and Williamson, G. (1998) FEBS Lett. 436, 71–75.
[16] Lambert, N., Kroon, P.A., Faulds, C.B., Plumb, G.W., McLauchlan, R.W., Day, A.J. and Williamson, G. (1999) Biochim. Biophys. Acta (in press).
[17] Rivera-Sagredo, A., Cañada, F.J., Nieto, O., Jimenez-Barbero, J. and Martín-Lomas, M. (1992) Eur. J. Biochem. 209, 415–422.
[18] Gee, J.M., DuPont, M.S., Rhodes, M.J.C. and Johnson, I.T. (1998) Free Radical Biol. Med. 25, 19–25.
[19] Wilkinson, G.N. (1961) Biochem. J. 80, 324–332.
[20] Price, K.R. and Rhodes, M.J.C. (1997) J. Sci. Food Agric. 74, 331–339.
[21] Withers, S.G., Street, I.P., Bird, P. and Dolphin, D.H. (1987) J. Am. Chem. Soc. 109, 7530–7531.
[22] Cañada, F.J., Díaz, J.C., García, A. and Martín-Lomas, M.

(1998) XIX International Carbohydrate Symposium, CP 084, EEUU, San Diego, CA.
[23] DuPont, M.S., Gee, J.M., Price, K.R. and Johnson, I.T. (1999) Gut 44 (Suppl. 1), A130.
[24] Fernandez, P., Cañada, F.J., Jiménez-Barbero, J. and Martín-Lomas, M. (1995) Carbohydr. Res. 271, 31–42.
[25] Hanke, D.W., Warden, D.A., Evans, J.O., Fannin, F.F. and Dietrich, D.F. (1980) Biochim. Biophys. Acta 599, 652–663.
[26] Hollman, P.C.H., de Vries, J.H.M., van Leeuwen, S.D., Mengelers, M.J.B. and Katan, M.B. (1995) Am. J. Clin. Nutr. 62, 1276–1282.
[27] Hirayama, B., Lostao, M.P., Panayotova-Heiermann, M., Loo, D.D.F., Turk, E. and Wright, E.M. (1996) Am. J. Physiol. 270, G919–G926.
[28] Spencer, J.P.E., Chowrimootoo, G., Choudhury, R., Debnam, E.S., Srai, S.K. and Rice-Evans, C. (1999) FEBS Lett. 458, 224–230.
[29] Crespy, V., Morand, C., Besson, C., Manach, C., Démigné, C. and Rémésy, C. (1999) SFRR Europe Winter Meeting: Bioflavonoids and Polyphenols in Health and Disease, Dinard, P002.
[30] Sathyamoorthy, N. and Wang, T.T.Y. (1997) Eur. J. Cancer 33, 2384–2389.