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THE ENZYMATIC DECOMPOSITION OF SALICIN AND ITS DERIVATIVES OBTAINED FROM SALICACEAE SPECIES

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ABSTRACT.—The enzymatic catalysis of the decomposition of Salicaceae phenolic glucosides was tested using almond β-glucosidase and rabbit and porcine liver esterases. The β-glucosidase catalyzed the complete hydrolysis of salicin and salicortin, yielding saligenin and glucose. Salicortin also produced (+)-6-hydroxycyclohexen-2-one (6-HCH). The acyl-glucosides were not decomposed by the β-glucosidase. Both esterases catalyzed the decomposition of tremulacin, salicortin, and 2′-*O*-acetylsalicortin, releasing tremuloidin, salicin, and 2′-*O*-acetylsalicin as the main products, accompanied by 6-HCH and catechol. Tremuloidin and 2′-*O*-acetylsalicin were quite stable under the esterase hydrolysis, and salicin was not decomposed at all.

Willows (*Salix* spp.) and poplars (*Populus* spp.) are both characterized by the existence of phenolic glycosides of low mol wt, such as salicin and its derivatives, which have been shown to be biologically active components in humans and insects. Salicin is an active precursor for salicyclic acid (1), and therefore willow bark, which contains a high amount of total salicin, is used in phytotherapy as an analgesic drug. Salicortin and tremulacin have been reported to be defensive components against herbivore feeding (2).

The metabolic fate of salicin and its naturally occurring derivatives, such as salicortin, tremulacin (3), 2′-*O*-acetylsalicortin (4), and 2′-*O*-acetylsalicin (5) in insects and humans has not been fully investigated. The bioavailability of salicin has been studied by Steinegger and Hövel (6) and Penz *et al.* (7) with respect to human beings and by Fötsch *et al.* (8) with respect to rats. Steinegger and Hövel (6) and Fötsch *et al.* (8) used pure salicin while Penz *et al.* (7) tested a herbal remedy that contained 166 mg of willow extract standardized to 11% of salicin per dosage. In both studies, salicylic acid was observed in plasma less than 60 min after digestion. The mechanism of aglucosidation to saclicyl alcohol and further oxidation to salicylic acid is not known. It has been suggested, on the basis of a study of germ-free rats, that the β-glucosidases of intestinal flora mainly react with salicin (9). The fast kinetics of the metabolism of salicin in human beings, however, indicate the probability of further metabolic pathways.

A different metabolism of salicin has been observed in some insects. The larvae of *Phratora vitellinae* (Chrysomelinae) use metabolites for their own defense by converting salicin to salicylaldehyde (10). This transformation occurs in defense glands, where the β-glucosidase activity is four times higher than that in the gut. The released glucose moiety is probably then used as an excess energy source. Recently, it has been reported that the midgut β-glucosidases of certain subspecies of the eastern tiger swallowtail (*Papilio glaucus*) can metabolize salicin and salicortin (11). Only glucose was analyzed as a resulting product. It was suggested that the hydrolysis of salicin yields glucose and salicyl alcohol, and that the hydrolysis of salicortin yields glucose and a "cyclohexenone saligenin ester." The latter suggestion is contrary to earlier chemical studies, in which alkaline hydrolysis (at pH more than 7) of salicortin yielded salicin (1,3,12). The 1-hydroxy-6-oxo-2-cyclohexen-1-carbonyl moiety of salicortin and tremulacin is easily liberated. Spontaneous decarboxylation then gives (+)-6-hydroxycyclohexen-2-one (6-

HCH) as a decomposition product (13). However, in acidic conditions (e.g., in artificial gastric juice) down to pH = 1.0, salicortin has been found to be quite stable (14).

This research was carried out to examine the substrate specificity of β-glucosidases and esterases from two different sources for phenolic glycosides which are ecologically and phytotherapeutically important. The main emphasis was on the hydrolytic pathways of the compounds, which have an acylated substituent in a sugar moiety. In tremulacin and tremuloidin this kind of substituent is a benzoyl group and in acetylsalicin

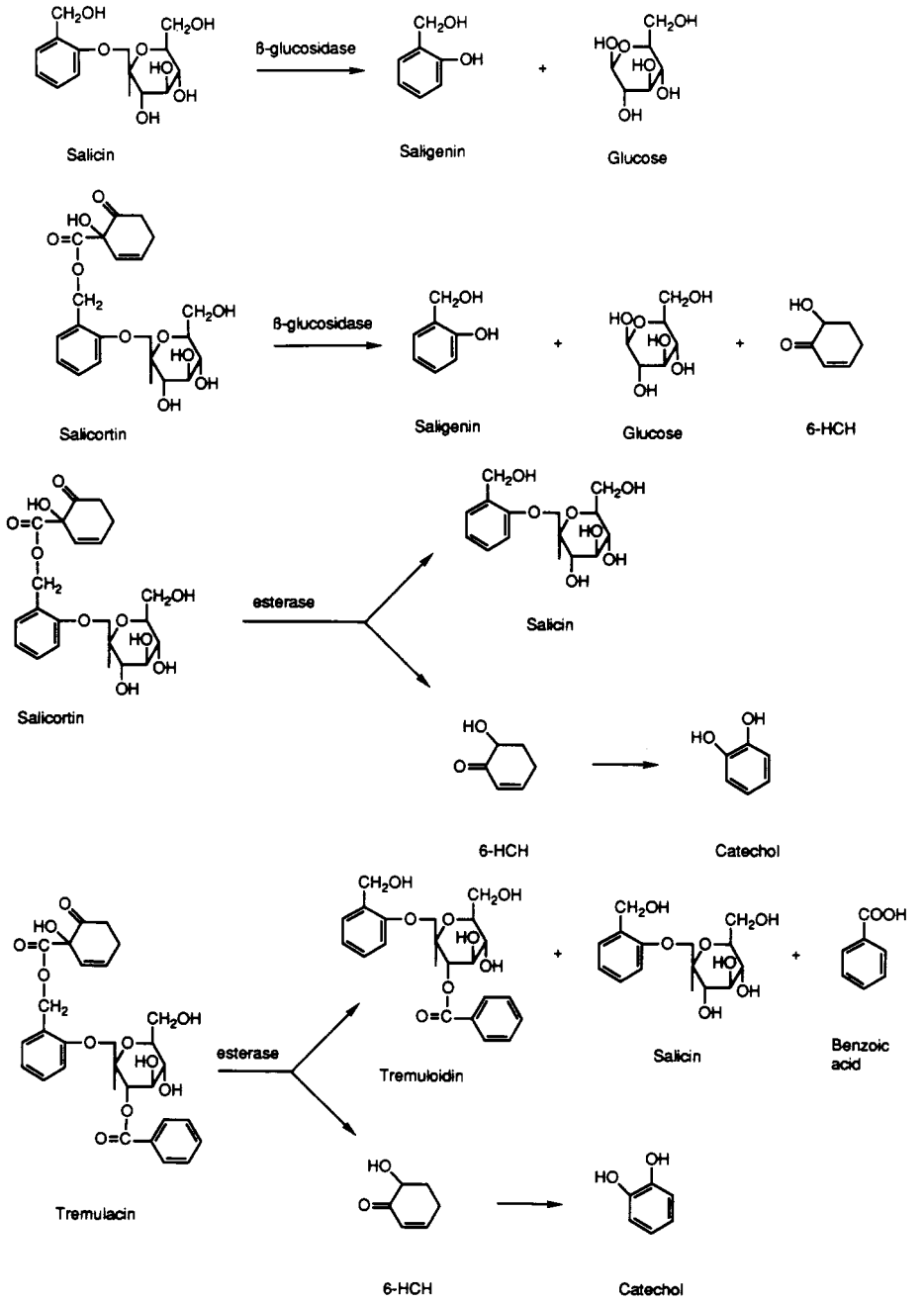

FIGURE 1. The β-glucosidase and esterase catalyzed hydrolysis of phenolic glucosides.

and acetylsalicortin it is an acetyl group. We propose that β-glucosidases cannot liberate a glucose from the willow glucosides, which contain an acylated sugar moiety. Moreover, we propose that nonspecific esterases may liberate the acyl group from the sugar moiety. Earlier, enzyme preparations obtained from *Populus balsamifera* (Salicaceae) (13) have been shown to decompose salicin and salicortin liberating 6-HCH from salicortin. Recently, Clausen *et al.* (15) described an unusual fragmentation and recombination by a β-glucosidase-catalyzed hydrolysis of salicortin. They postulated a fragmentation of salicortin into orthoquinone methidine, CO_2, and an enol. The described enol is a tautomer of 6-HCH. The enol and methidine are recombined to 6-hydroxy-6-(2′-hydroxybenzyl)-cyclohexenone. Moreover, picein, the naturally occurring glucoside, has been reported to support high rates of hydrolysis by the broad-specificity β-glucosidases obtained from guinea pig liver (16), but there are no studies of the catalytic effect of pure animal esterases on the decomposition of willow-derived phenolic glucosides.

RESULTS AND DISCUSSION

THE METABOLISM BY THE β-GLUCOSIDASE.—The decomposition of salicin, 2′-*O*-acetylsalicin, salicortin, 2′-*O*-acetylsalicortin, and tremulacin as a substrate was tested in vitro by using almond-β-glucosidase. Our studies revealed that the β-glucosidase is quite a specific enzyme against willow glucosides. It decomposed only salicin and salicortin, the compounds of which have a free sugar moiety (Figures 1 and 2). The hplc analyses showed a yield of 100% of the expected amount of saligenin for salicin and more than 97% for salicortin (Table 1). Similarly, the gc analysis of glucose released showed 100% of the amount expected. In the reaction mixture of salicortin, the hplc analysis also indicated the presence of two extra peaks (Figure 3), which could be the decomposition counterparts of salicortin. The comparison of the chromatographic properties of one of these peaks with authentic 6-HCH gave a similar low retention time (Rt 0.65) and a spectral-matching index between 986 and 994 (maximum value = 1000) (Figure 2), indicating that the esterically bonded salicortin counterpart is easily broken down during β-glucosidase hydrolysis. The presence of saligenin and 6-HCH after the β-glucosidase reaction was also verified by gc and gc-ms analyses, respectively. The fragmentation of 6-HCH in the hydrolysate was equal to the 6-HCH reference compound [ms *m/z* 112, 94, 84, 68 (100%)]. The results are in agreement with the work of Mattes *et al.* (13), who reported that, during extraction, salicortin releases 6-HCH and this process is enzyme-catalyzed. Our results seem to be contradictory to the findings of Clausen *et al.* (15). However, both sets of products apparently

TABLE 1. The Hydrolysis of Phenolic Glucosides by β-Glucosidases.[a]

Compound	Hydrolyzed products (% of the expected)	
	Saligenin	Glucose
Salicin	100.64 (1.07)	100.04 (2.38)
Salicortin	97.54 (4.10)	104.85 (0.84)
Acetylsalicin[b]	no decomposition	
Acetylsalicortin[b]	no decomposition	
Tremulacin[b]	no decomposition	

[a]The values represent the % of the expected amount, and SE is shown in parentheses.

[b]Acylation in the sugar moiety blocks the activity of the specific β-glucosidases.

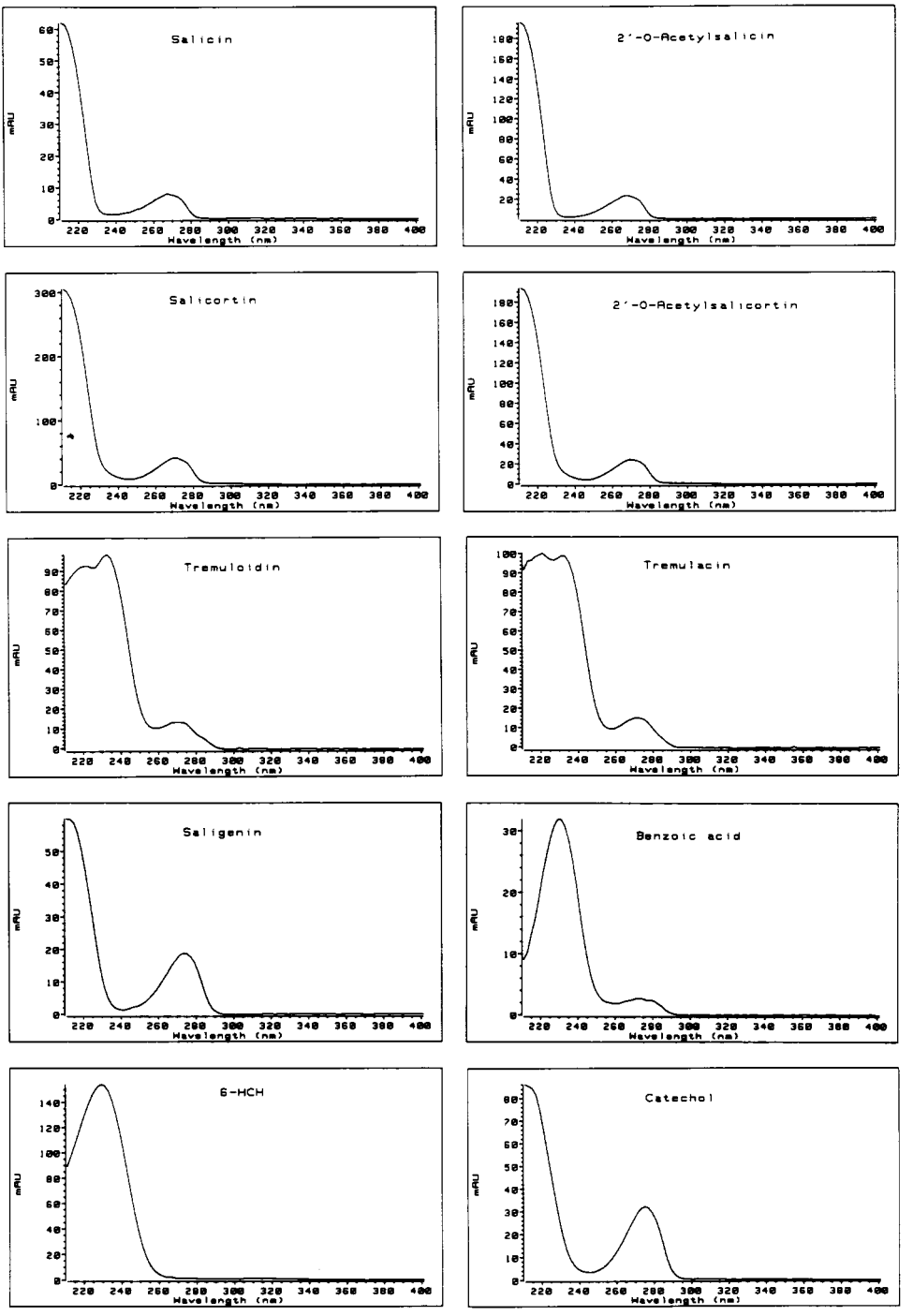

FIGURE 2. The uv spectra of the willow (*Salix*) phenolics tested in the enzymatic experiments and their decomposition products.

come from fragmentation of salicortin to 1 and 2 with different modes of reaction of 1 and 2 leading to the products observed by Clausen *et al.* (15), and ourselves (Figure 4). The pathway to saligenin and 6-HCH can be explained by the addition of H_2O to the orthoquinone methidine [**1**] instead of the recombination of 1 and 2 to form the major

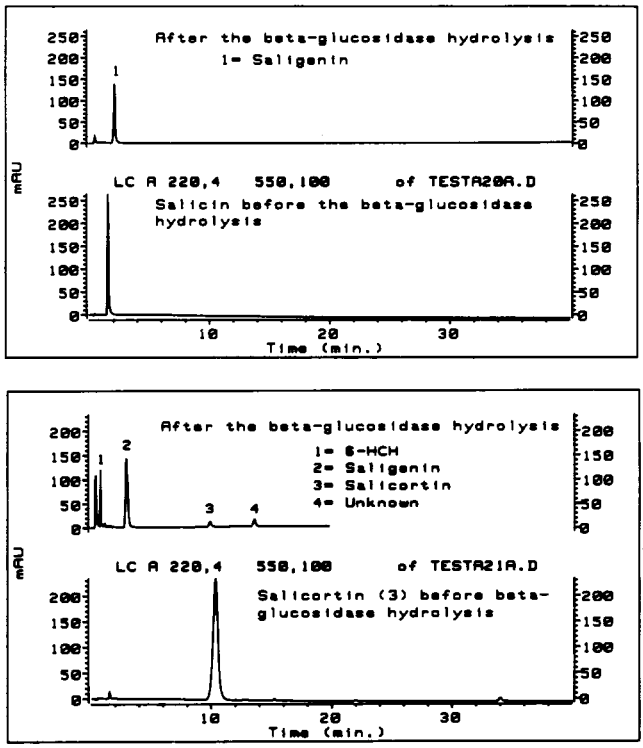

FIGURE 3. The hplc traces of the decomposition of salicylates by β-glucosidases.

end product of Clausen *et al.* (15). Different experimental conditions may cause different end products. Moreover, our results indicate that 6-HCH is quite stable in the acidic conditions needed for β-glucosidase activity, because no phenols or catechols were found.

On the other hand, 2′-*O*-acetylsalicin, 2′-*O*-acetylsalicortin, and tremulacin were not decomposed by the β-glucosidase; the chromatograms were identical before and after enzymatic reaction. The acetyl and benzoyl moieties attached to the glucose molecule completely hindered the action of β-glucosidase. This contradicts previous ideas about the catalytic activity of β-glucosidase (17). Our results also showed the high stability of acylated derivatives of salicin, such as tremulacin, 2′-*O*-acetylsalicin, and 2′-*O*-acetylsalicortin, in acidic environments, since the compounds hydrolyzed in a control mixture (a buffer, pH 5.0 without β-glucosidases) were not changed during the reaction.

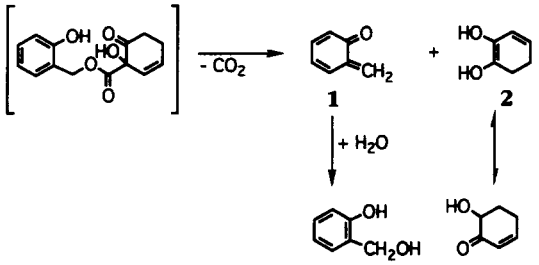

FIGURE 4. The theoretical reaction scheme for the decomposition of the aglycone of salicortin.

It has been claimed that while the β-glucosidase is specific for β-glucopyranosides, it is relatively non-specific as regards the aglycone moiety, which means that it will hydrolyze most classes of phenolic glycosides, except the anthocyanidins (18). This is also true in the case of willow phenolic glucosides, but the β-glucosidase activity depends on the substitution of the glucose moiety.

THE METABOLISM BY THE ESTERASES.—The decomposition of salicin, 2′-*O*-acetylsalicin, salicortin, 2′-*O*-acetylsalicortin, and tremulacin was studied using two esterases from different animal sources. These enzymes function in alkaline conditions (pH = 7.5 and 8.0), which may itself affect the decomposition of certain willow glucosides. The effect of a high pH on phenolic glucosides was tested by keeping a control sample under the same conditions without enzymes; a few small unknowns appeared, indicating some decomposition. This chemical decomposition was minimized by acidifying the samples to pH 6 after the enzymatic hydrolysis.

Neither of the esterases was able to catalyze decomposition of salicin. This was also confirmed by gc analyses, which showed the absence of glucose, salicyl alcohol, or unknowns. Salicortin, on the other hand, was readily decomposed to salicin and 6-HCH by both esterases (Figures 1 and 5). 6-HCH was partially converted by oxidation to catechol. The decomposition by rabbit liver esterase was complete after 40 min hydrolysis, and less than 2% of salicortin was left after porcine liver esterase catalysis (Table 2). Tremulacin was completely decomposed, producing mainly tremuloidin, but also salicin accompanied with benzoic acid, 6-HCH, catechol, and an unknown, which had a uv spectrum similar to that of tremuloidin (Figure 5). The weakest ester bond, the cyclohexenonol linkage, was broken down, as was earlier the case in alkaline hydrolyses (1,3) and in our β-glucosidase tests for salicortin. In our study, tremuloidin was not found to be easily decomposed by esterases, which may be due to the benzoyl moiety of the glucose part of the molecule. It is interesting to note that populin, which is a chemical isomer of tremuloidin with a benzoylated glucose moiety, has also found to be very stable in human beings (6). 2′-*O*-Acetylsalicortin was decomposed almost completely by rabbit liver esterase to 2′-*O*-acetylsalicin and, to a lesser extent, to salicin, releasing 6-HCH and catechol (Table 2, Figure 5). The porcine liver esterase acted similarly, but about 5% of 2′-*O*-acetylsalicortin was still left after hydrolysis. Also an unknown component with a uv spectrum similar to that of 2′-*O*-acetylsalicin appeared after the esterase hydrolysis of 2′-*O*-acetylsalicortin. On the other hand, it was difficult to achieve complete decomposition of 2′-*O*-acetylsalicin using esterases. After 40 min hydrolysis, only about 30% of 2′-*O*-acetylsalicin was changed, producing mainly the same unknown salicylate found after the esterase hydrolysis of 2′-*O*-acetylsalicortin. Some 2′-*O*-acetylsalicin was also changed to salicin (Table 2).

In conclusion, the animal liver esterases used in this study readily freed the 6-HCH moiety from salicylates, but further decomposition so that the acyl moiety separated from the molecule was more difficult to achieve. Acylation of the sugar moiety hindered the catalytic activity of the esterases. On the other hand, β-glucosidase was able to decompose only salicin and salicortin, which have a free glucose moiety in the molecule, and β-glucosidase activity was totally blocked by the acyl group linked with glucose.

EXPERIMENTAL

β-Glucosidase (EC 3.2.1.21, Sigma Chemical Co.) extracted from almonds and esterases obtained from rabbit and porcine liver (EC 3.1.1.1, Sigma Chemical Co.) were used to hydrolyze salicin, 2′-*O*-acetylsalicin, salicortin, 2′-*O*-acetylsalicortin, and tremulacin as a substrate. Salicin was obtained from Sigma Chemical Co. All the other glucosides were extracted and purified from different willow species (4,5,14). 6-HCH was kindly supplied by Prof. Paul Reichardt, University of Alaska. Phosphate buffers (KH_2PO_4

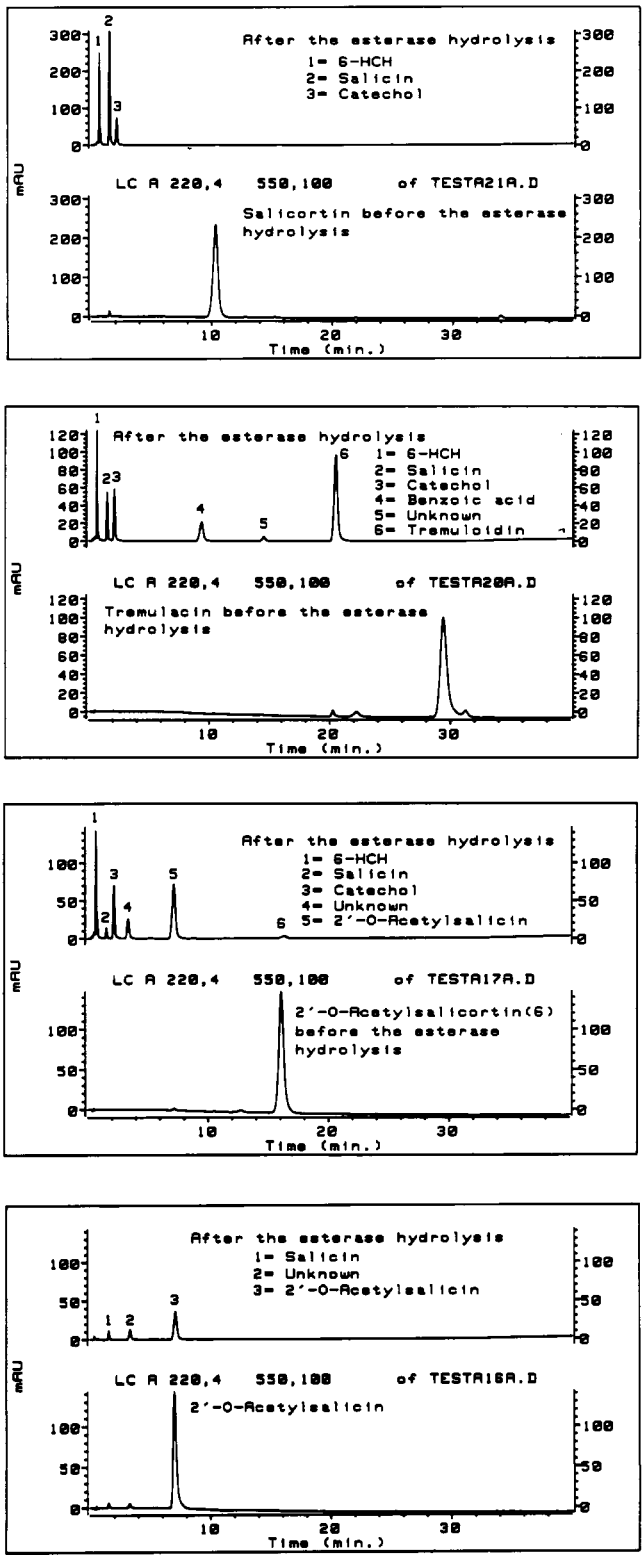

FIGURE 5. The hplc traces of the decomposition of salicylates by esterases.

TABLE 2. The Hydrolysis of Phenolic Glucosides by Rabbit and Porcine Liver Esterases.[a]

Hydrolysis product	Rabbit liver esterase	Porcine liver esterase
Salicortin hydrolysis:		
Salicin	98.12(0.05)	93.81(0.13)
Salicortin	0	1.61(0.13)
Tremulacin hydrolysis:		
Salicin	18.70(1.1)	25.35(1.1)
Tremuloidin	63.93(1.4)	62.55(0.71)
Unknown 1	10.17(0.41)	2.66(0.08)
2′-*O*-Acetylsalicortin hydrolysis:		
Salicin	3.61(0.1)	4.61(0.12)
Acetylsalicin	75.47(0.25)	69.81(0.20)
Acetylsalicortin	0	4.72(0.56)
Unknown 2	15.96(0.31)	17.11(0.30)
2′-*O*-Acetylsalicin hydrolysis:		
Salicin	6.53	10.02(1.4)
Acetylsalicin	70.68	67.72(2.9)
Unknown 3	19.30	19.19(0.68)

[a]The values represent the % of the expected amount, and SE is shown in parentheses. Unknowns 1, 2, and 3 are calculated using tremuloidin, acetylsalicortin, and acetylsalicin, respectively, as standards. Acylation in the sugar moiety markedly affected the activity of both esterases.

and Na_2HPO_4) at pH = 5.0, pH 7.5, and pH 8.0 were used for the β-glucosidase, the rabbit liver esterase, and the porcine liver esterse activity tests, respectively.

In vitro activity measurements for β-glucosidase and esterases were assayed by measuring the glucose and salicyl alcohol freed from salicin and 2′-*O*-acetylsalicin, and the glucose, salicyl alcohol, salicin, 6-HCH, tremuloidin, populin, catechol, and benzoic acid freed from salicortin, 2′-*O*-acetylsalicortin, and tremulacin. The reaction mixture (final volume 0.5 ml), which was standardized by preliminary assays, consisted of 10 U β-glucosidase (4.8 units/mg protein), 22 U rabbit liver esterase (100 U/mg protein), and 25 U procine liver esterases (230 units/mg protein) and 0.5–0.9 μmol of the glucoside to be studied. All the glucosides were assayed separately with three replicates (except two replicates were done in rabbit liver esterase tests of 2′-*O*-acetylsalicin). The hydrolyses were conducted at 37° for 20 min for the β-glucosidase and at 25° for 40 min for the esterases. After esterase hydrolysis, the pH of the reaction mixture was acidified to prevent glucoside decomposition under alkaline conditions. Glucosides in a phosphate buffer served as a control measurement.

Hplc was used for the analyses of phenolic glucosides and their hydrolysis products, using a method published previously (19). An aliquot of each hydrolyzed mixture was filtered with an RP-C_{18} column, evaporated, and derivatized with trimethylsilylimidazole in pyridine (20). TMSi samples were used for the quantification of the released glucose and for the confirmation of the existence of all other products of hydrolysis by capillary gc. A programmed run was started at 150°, followed by a rise of 8°/min to 295°. The injector and detector temperature were 230 and 300°, respectively. A fused silica OV- 1 capillary column (25 m in length with a phase layer of 0.25 μm) was used throughout. He was used as the carrier gas, the split ratio was 1:15, and the injected volume 1μl. Gc-ms for 6-HCH was processed as published previously (21). Since the preliminary studies showed that about 50% of the glucose was bound to the β-glucosidase, the control glucose standard was determined by hydrolyzing the glucose with an equal amount of the active β-glucosidase used the enzymatic tests.

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