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Note

Hydrogen isotope fractionation during the hydrolysis of starch and the fermentation of D-glucose to ethanol

GÉRARD J. MARTIN*, BENLI ZHANG,

Université de Nantes, Laboratoire de RMN et Réactivité Chimique UA, CNRS 472, 2 rue de la Houssinière, F-44072 Nantes (France)

LUC SAULNIER, AND PAUL COLONNA

Institut National de la Recherche Agronomique, Laboratoire de Biochimie des Glucides, La Géraudière, F-44072 Nantes (France)

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Starch is the main reserve carbohydrate in higher plants and one of the major sources for fermentation processes[1]. Although the fate of the carbon atoms during photosynthesis and glycolysis is now well documented, less is known about the fate of the hydrogen atoms. Stable isotope analysis at the natural abundance level has been used extensively in this context[2] but, due to their lack of specificity, the average isotope ratios ($\overline{D/H}$) obtained by mass spectrometry do not provide unambiguous mechanistic answers. ^{2}H-N.m.r. spectroscopy has been used[3] to determine site-specific natural-isotope fractionation factors (SNIF-NMR) and is useful for analytical purposes in beverage identification[4]. Direct determination of the pattern of deuterium distribution in amylose and amylopectin by this method is not practicable at present for technical reasons. However, since significant correlations have been found between the SNIF-ratios measured in ethanols and the deuterium content of the starting D-glucose, the question arises as to whether ethanol can be used as a probe for characterising the isotope contents of starches.

Acid hydrolysis[5] involves cleavage of the C-1–O-1 bond with an electron deficiency at C-1 in the transition state, and several by-products can be formed[5]. Nevertheless, a quantitative hydrolysis of starch[6] is achievable[7]. The enzymic hydrolysis involves[8] acid and base assistance at C-1 and the resulting acidity of the C-1–H-1 bond should be less than in purely acidic conditions. Differences in the deuterium distribution in the resulting ethanols can be characterised[3] by the relative parameter, R, and the absolute site-specific isotope ratios $(D/H)_i$.

The specific isotope ratios of ethanol produced from various starches will reflect isotope contents of the starting materials only if differential isotope effects, possibly associated with variations in the yield of D-glucose, are neglected or can be controlled. Therefore, the effect of varying the concentration of substrate, pH, and

*To whom correspondence should be addressed.

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isotope content of the starting water in acid and enzymic hydrolyses has been studied.

Increase in the initial concentration of starch results in a lower yield of D-glucose especially in acid hydrolysis. A nearly quantitative transformation (98%) of starch can be obtained in 4 h in acid hydrolysis with an initial concentration of 0.35%, whereas a similar yield can be obtained with a 1% solution by enzymic hydrolysis. For enzymic hydrolysis, the yield decreased to 89.3% for a 4% solution. An increase in the pH also produced a significant decrease in the yield of the enzymic hydrolysis (93.8% at pH 5.5, 98% at pH 4.65). The natural origin of the starch had only a slight effect on the yield of D-glucose.

For a given type of hydrolysis, there was no significant effect on the isotope ratios on varying the experimental conditions, but the method (acidic or enzymic) did have an effect (Table I). Thus, on using water with an initial deuterium content of 148.0 p.p.m., the isotope ratio $(D/H)_W$ was ~157 p.p.m. after acid hydrolysis and ~151.5 p.p.m. after enzymic transformation (Table I). Moreover, enzymic and acid hydrolyses are characterised by different isotope fractionations in the resulting

TABLE I

DISTRIBUTION OF DEUTERIUM IN ETHANOL DERIVED FROM VARIOUS STARCHES

Origin of starch	*Hydrolysis*	*Fermentation medium*[a]		*Isotope parameters*[b]			
		pH	$(D/H)_W$	*R*	$(D/H)_I$	$(D/H)_{II}$	$\overline{D/H}$
Cereals							
Maize	Acid	2.4	158.7	2.27	113.0	125.6	125.5
	Enzyme	3.4	151.2	2.11	111.6	120.4	121.8
Wheat	Acid	2.6	158.3	2.56	98.3	122.4	117.0
	Enzyme	3.9	152.7	2.46	98.0	121.7	115.6
Rice	Acid	2.5	156.6	2.55	98.1	121.1	116.2
	Enzyme	3.4	151.3	2.41	98.0	116.9	113.8
Tuber							
Potato	Acid	3.0	156.0	2.64	94.4	123.3	115.0
	Enzyme	3.6	150.8	2.53	94.2	120.4	113.0
Manioc	Acid	2.4	160.0	2.61	—	—	—
	Enzyme	3.9	152.0	2.51	95.8	121.6	114.4
Leguminosae							
Pea	Acid	2.5	157.1	2.68	94.4	124.1	115.1
	Enzyme	4.2	151.2	2.58	94.2	123.0	113.9
Bean	Acid	2.6	157.0	2.60	95.9	124.0	116.1
	Enzyme	4.1	151.0	2.55	93.7	122.2	114.9

[a] $(D/H)_W$ is the isotope ratio (in p.p.m.) of water at the end of the fermentation. [b] R is the relative internal parameter derived from signal heights, $R = 3\ h_{II}/h_I$; $(D/H)_I$ and $(D/H)_{II}$ are the site-specific isotope ratios (in p.p.m.) of the methyl and methylene sites of ethanol. The average parameter $\overline{(D/H)}$ gives only an approximate trend of the overall deuterium variations, since the isotope ratio of the hydroxyl site is dependent on exchange with water and fractionation during the distillation. The confidence intervals (97.5%) are ±0.01 for R and ±1 p.p.m. for $(D/H)_i$. (External ref. 3c.)

ethanol obtained by fermentation of the resulting D-glucose with *S. cerevisiae*. The enzymic conversion is associated with a smaller value of the relative parameter R which describes[3] the isotope content of the methylene site, the methyl site being arbitrarily given the statistical value 3.

The results show that there is a transfer of deuterium from starch or D-glucose to the aqueous medium but no significant transfer during the hydrolysis step. In order to check this interpretation, maize and rice starches were enzymically hydrolysed in water having a D/H isotope ratio of 148 p.p.m. The resulting D-glucose was purified and then dissolved in water having the same D/H ratio. After fermentation, this value had risen to 151.5 p.p.m. Similar values (151.0 to 152.0 p.p.m.) were obtained when the fermentation was performed with the mother liquor from the hydrolysis. Therefore, deuterium transfer occurs from D-glucose to water and ethanol during fermentation rather than from starch to water during hydrolysis. This interpretation is further corroborated by the lack of sensitivity of the isotope parameter R of ethanol to the deuterium content (in p.p.m.) of the hydrolysis medium (Table II).

Thus, it is concluded that there is no deuterium exchange during the hydrolysis and that the systematic excess in deuterium transfer associated with the acid procedure is probably caused by the chemicals used in the neutralisation step. Also, the hydrolysis procedure has a very limited effect on the isotope content, $(D/H)_I$, of the methyl site. The smaller R value measured for the enzymic hydrolysis is therefore mainly the result of a smaller deuterium content, $(D/H)_{II}$, in the methylene site, which is associated with the smaller isotope ratio of the fermentation medium (Fig. 1). This conclusion is quantified by the correlations between the parameters of the acid [R^A, $(D/H)_I^A$] and enzymic [R^E, $(D/H)_I^E$] hydrolyses:

$$R^A\ (\pm 0.02) = 0.53\ (\pm 0.3) + 0.83\ (\pm 0.05)\ R^E \qquad (r = 0.99,\ n = 7)$$
$$(D/H)_I^A\ (\pm 0.9) = -2\ (\pm 1.3) + 1.029\ (\pm 0.05)\ (D/H)_I^E \qquad (r = 0.99,\ n = 6).$$

Although they can be slightly influenced by variations in the isotope content of the fermentation medium, the $(D/H)_I$ values of ethanol are representative of the origin of the starch. Thus, C3 plants such as wheat, rice, *etc.*, are characterised, as expected[3b], by smaller deuterium contents than C4 plants (maize). Therefore,

TABLE II

DEUTERIUM CONTENT (P.P.M.) OF THE HYDROLYSIS AND FERMENTATION MEDIA AND THE ISOTOPE PARAMETER OF THE RESULTING ETHANOL

$(D/H)_W$ hydrolysis	*$(D/H)_W$ fermentation*	*R (ethanol)*
149.9	149.9	2.48
	259.8	3.06
259.8	149.9	2.49
	259.8	2.97

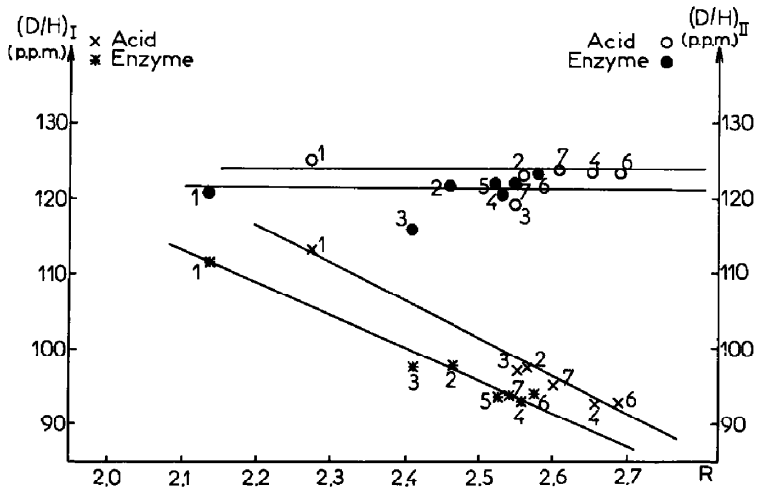

Fig. 1. Influence of the nature of the plant and of the mode of hydrolysis on the relative (R) and site-specific [$(D/H)_i$] isotope parameters of ethanol derived from 1, maize; 2, wheat; 3, rice; 4, potato; 5, manioc; 6, pea; and 7, bean.

under controlled experimental conditions, ethanol obtained after acid or enzymic hydrolysis can be used as a probe for characterising starches from various botanical species.

EXPERIMENTAL

Materials. — Starches from various botanical sources were extracted and purified according to described procedures[7b]

Hydrolysis. — (*a*) *In acid.* Starch (50 g/L) was hydrolysed[7a] at 100° in 0.35M sulfuric acid for 3 h. The solution was then neutralised and concentrated, and the residue was purified.

(*b*) *Enzymic.* Starch (5 g) was gelatinised in water (100 mL) and heated at 135° (2.5 bar). The final volume was adjusted to 242.5 mL with water. Acetate buffer (pH 4.8) and a solution of amyloglucosidase were added[9]. The hydrolysis was carried out at 60°. The solution was then concentrated and the residue was purified.

The D-glucose formed in (*a*) and (*b*) was determined[10] enzymically. The overall concentration of reducing sugars was measured[11], and the proportions of the various oligosaccharides were determined[12] by h.p.l.c.

Fermentation. — The concentration of the hydrolysate was adjusted to 100 g/L and the yeast *(Saccharomyces cerevisiae)* was used in a synthetic medium at pH 7. The medium was first sterilised at 120° for 20 min, and the added solution of vitamins was sterilised by ultrafiltration. The fermented solutions were then distilled and the n.m.r. spectra were recorded under standardised conditions[4].

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