Enzymic deacetylation of the peracetylated xylosides in anhydrous methanol

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Dedicated to Professor P. Hrnčiar, DrSc., in honour of his 60th birthday

Using serine hydrolases such as trypsin, chymotrypsin, pancreatic lipase and plasmic pseudocholinesterase as the catalysts, a study of the enzymic deacetylation of methyl and umbelliferyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranosides in anhydrous methanol was carried out. Analysis of the products was performed by means of thin-layer chromatography on silica gel. It was found that the mentioned enzymes catalyzed deacetylation of the peracetylated xylopyranosides in anhydrous medium. However, deacetylation was inselective, and no increase of the selectivity was reached even when arginine, tyrosine, glycerol, and choline were used as the acceptors of the leaving acetyl groups.

Изучено ферментативное деацетилирование метил- и умбеллиферил-2,3,4-три-*O*-ацетил-β-D-ксилопиранозида в безводном метаноле с использованием сериновых гидролаз: трипсина, химотрипсина, липазы из поджелудочной железы и псевдохолинэстеразы из плазмы крови в качестве катализаторов. Анализ продуктов производился с помощью тонкослойной хроматографии на силикагеле. Найдено, что перечисленные ферменты катализируют деацетилирование перацетилированных ксилопиранозидов в безводной среде. Деацетилирование, однако, протекает неселективно, и повышения избирательности не удалось достичь даже если в качестве акцепторов отщепляемых ацетильных групп были использованы аргинин, тирозин, глицерин и холин.

It was assumed initially that enzymes were able to catalyze chemical reactions only in aqueous solutions or in the presence of water. It was not until the beginning of the eighties when several reports on the enzymic catalysis in anhydrous organic solvents have appeared in the literature [1—5]. If one uses an enzyme in the anhydrous medium, it is possible to observe significant increase of its thermostability [6] and sometimes even the change in its substrate specificity [7].

One of the most important factors determining behaviour of an enzyme in anhydrous organic solvent is a choice of the appropriate solvent [8]. It was

found that the more hydrophobic was the solvent, the more adequate it was for the enzymic catalysis in anhydrous media [5]. Similarly, stability of the enzyme was also determined by the nature of the solvent [6]. Depending on the nature of the solvent, large differences were found also for the values of the relative enzymic activity [3, 9]. Recent investigations of the transesterification reactions in various organic solvents catalyzed by subtilisine and chymotrypsin demonstrated that the values V_{max}/K_m approximately correlated with the hydrophobicity of the solvent [10]. It follows from this paper that the solvents with higher hydrophobicity are more appropriate for the enzymically catalyzed transesterification reactions than the hydrophilic, miscible with water solvents.

As yet only few studies have been made in which enzymically catalyzed transesterification reactions in the anhydrous media had been applied to the purposive modification of the saccharides. *A. M. Klibanov et al.* managed in 1986 to carry out regioselective acylation of the monosaccharides in the position 6 of the pyranose ring by means of the lipases in the anhydrous dimethylformamide, *i.e.* in the polar aprotic solvent, in which enzymic catalysis was not as yet successful [11, 12]. Although selective deacetylation of the peracetylated glycosides also belongs to the important reactions of the synthesis of saccharides and their derivatives [13], its enzymic catalysis has been studied so far only in aqueous or mixed water-organic media [14—17].

In this work we studied an enzymically catalyzed deacetylation of peracetylated methyl and umbelliferyl β-D-xylosides in nonaqueous hydrophilic protic medium — anhydrous methanol. As enzymic catalysts serine hydrolases trypsin and chymotrypsin were used, as well as horse plasmic cholinesterase and lipase from porcine pancreas.

Experimental

Trypsin (EC 3.4.21.4) from bovine pancreas was purchased from Fluka, Switzerland, chymotrypsin (EC 3.4.21.1) was from Medika, Bratislava, pseudocholinesterase (EC 3.1.1.8) from horse plasma was isolated by means of fractional precipitation and gel filtration on Sephadex G-200, lipase (EC 3.1.1.3) from porcine pancreas was a product of Sigma, USA.

Methanol (Lachema, Brno), pure, dried with magnesium contained less than 0.5 % water determined by Fischer's method [18].

Umbelliferyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (m.p. = 155—156 °C, $[\alpha](D, CHCl_3) = -42.8°$) and umbelliferyl β-D-xylopyranoside (m.p. = 212—213 °C, $[\alpha](D, pyridine) = -21.1°$) were prepared according to [19], methyl β-D-xylopyranoside (m.p. = 156—157 °C, $[\alpha](D, water) = -63.9°$) was prepared according to [20]. Partially acetylated derivatives of umbelliferyl and methyl β-D-xylopyranosides were obtained according to [21]. Silica gel plates for thin-layer chromatography of the reaction products

(Silufol) were from Kavalier, Sázava. Other reagents of anal. grade were from Lachema, Brno.

The enzymes were prior to the use dissolved in buffer solutions to produce a concentration $\varrho = 10\ \mathrm{mg\ cm^{-3}}$ as follows: trypsin was dissolved in 0.01 M Tris—HCl, pH = 8.2; chymotrypsin in 0.01 M Tris—HCl, pH = 7.6; pseudocholinesterase in 0.01 M Tris—HCl, pH = 7.4 and lipase in 0.1 M glycine—HCl, pH = 9.0. Enzyme solutions were frozen and lyophilized. The enzymes were further dried in the desiccator at the pressure *ca.* 20 kPa over P_2O_5 for at least 24 h.

Determination of enzyme activities was carried out as follows: of trypsin — by means of Bio-La-Test (Lachema, Brno) according to [22]; of chymotrypsin — by means of S-Test for proteases (J. Dimitrov Chemical Works, Bratislava); of plasmatic pseudocholinesterase — by *Ellman*'s method according to [23]; of lipase using olive oil as a substrate according to [24].

Specific activity of the enzymes established by means of the aforementioned methods before the use of the enzymes in the reaction was following (calculated for molar amount of the product formed at the given experimental conditions): trypsin 49.75 $\mu\mathrm{mol\ min^{-1}\ mg^{-1}}$, chymotrypsin 93670 U (units according to the manufacturer of the test kit, 1 U corresponds to 1 mg of the chromolytic substrate hydrolyzed during 1 h at 40 °C in 0.01 M phosphate buffer, pH = 7.6), pseudocholinesterase 4.74 $\mathrm{nmol\ s^{-1}\ mg^{-1}}$, lipase 6.62 $\mathrm{mmol\ min^{-1}\ mg^{-1}}$.

Deacetylation of the peracetylated β-D-xylosides was performed as follows. Xyloside (10^{-5} mol) was dissolved in 2 $\mathrm{cm^3}$ of methanol, 10—20 mg of dry lyophilized enzyme were added and the mixture was stirred at 48 °C in the water bath with a magnetic stirrer. In the case when more specific acetyl acceptor was used (arginine for trypsin, tyrosine for chymotrypsin, choline iodide for pseudocholinesterase, and glycerol for lipase), its amount was five times molar amount of the substrate.

For the separation of methyl β-D-xylopyranosides TLC was used in the system ethyl acetate—hexane ($\varphi_r = 4 : 1$). Detection was carried out using 5 % H_2SO_4 in methanol at 105 °C. For the separation of the derivatives of umbelliferyl β-D-xylopyranosides elution system ethyl acetate—benzene—hexane ($\varphi_r = 3 : 1 : 1$) was used and the detection was carried out by UV illumination at the wavelength 254 nm.

Results and discussion

Partially deacetylated xylosides are intermediates for the synthesis of the chromogenic substrates for xylanases and xylosidases. A possibility of their preparation by partial deacetylation of the peracetylated xylosides led us to use these compounds as the models for a study of the enzymically catalyzed hydrolysis in the nonaqueous media.

Enzymically catalyzed deacetylation of the *O*-peracetylated β-D-xylose was primarily tested with its umbelliferyl glycoside, trypsin having been used as a catalyst in anhydrous methanol (Fig. 1). Methanol served not only as a solvent, but also as an acceptor of the acetyl group. As can be seen in the figure, all

positions of xylose glycoside are deacetylated, none of them being preferred. The reaction does not take place at the same conditions in the absence of the enzyme.

We made an attempt to achieve selectivity of the trypsin-catalyzed deacetylation of umbelliferyl 2,3,4-tri-*O*-acetyl-β-D-xylose by adding L-arginine as a natural acceptor of acetyl group in the trypsin-catalyzed transesterification reac-

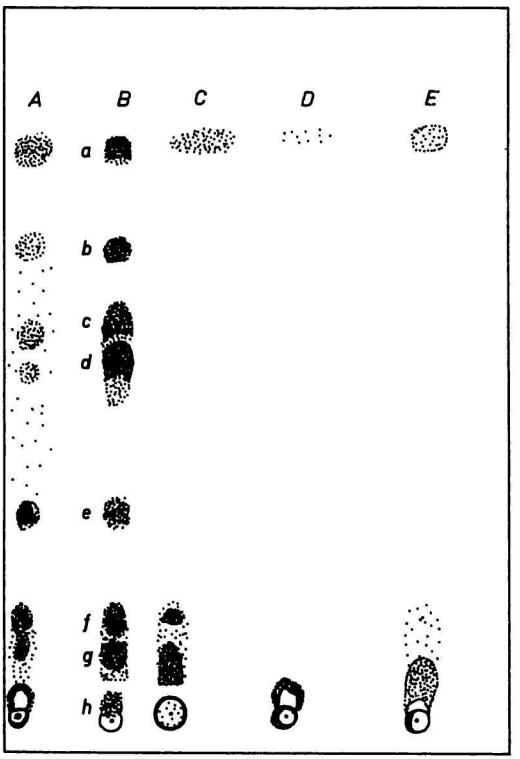
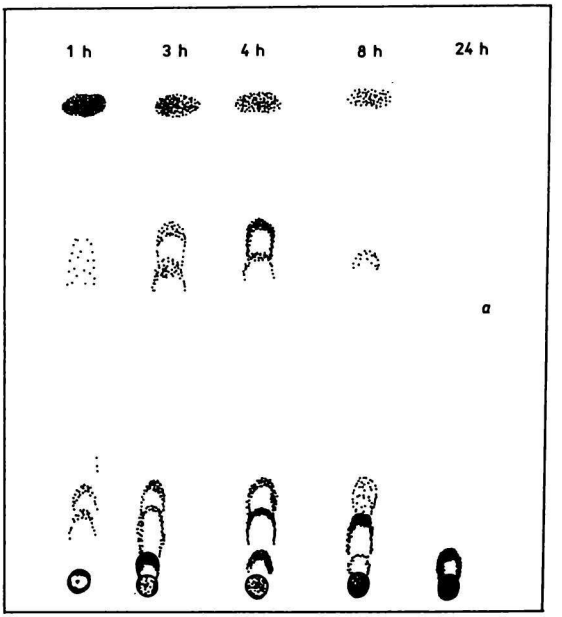
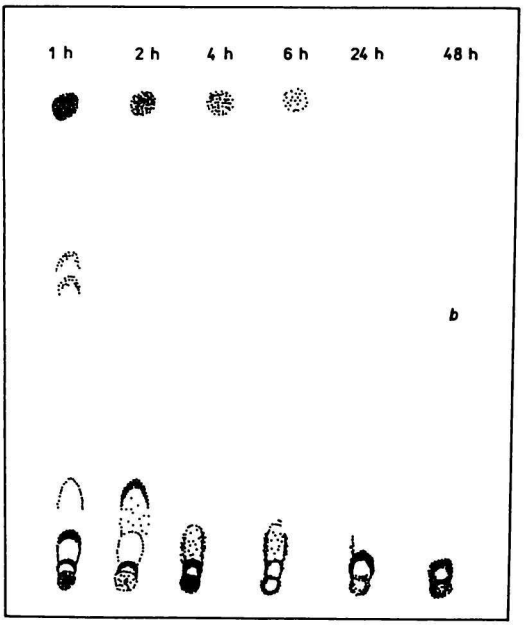


Fig. 1. Trypsin-catalyzed deacetylation of umbelliferyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (10^{-5} mol) in anhydrous methanol. *A*. Without arginine, reaction time 80 h. *B*. Standard mixture consisting of an equimolar mixture: *a*) original substrate; *b*) 3,4-di-*O*-acetyl-β-D-xylopyranoside; *c*) 2,3-di-*O*-acetyl-β-D-xylopyranoside; *d*) 2,4-di-*O*-acetyl-β-D-xylopyranoside; *e*) 3-*O*-acetyl-β-D-xylopyranoside; *f*) 4-*O*-acetyl-β-D-xylopyranoside; *g*) 2-*O*-acetyl-β-D-xylopyranoside; *h*) free β-D-xylopyranoside. *C*. Deacetylation in the presence of arginine (5×10^{-5} mol) in a reaction mixture without trypsin for 24 h. *D*. Trypsin-catalyzed deacetylation in the presence of arginine (5×10^{-5} mol). *E*. Incubation of the substrate without arginine in 50 vol. % system containing 0.01 M Tris—HCl buffer, pH = 8.2, and methanol.

tions. As seen from Fig. 1, arginine itself accelerates nonselective deacetylation of the derivative. Trypsin causes the increase of the rate of acetyl cleavage, but no selectivity for any position can be seen, which is better evident in Fig. 2*a*. We could not reach selectivity even in partially aqueous medium containing 50 vol. % 0.01 M Tris—HCl buffer solution with pH = 8.2, although the rate of deacetylation increased (Figs. 2*a* and 2*b*). In both cases all possible deacetyla-

Fig. 2. Time course of the trypsin-catalyzed deacetylation of umbelliferyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (10^{-5} mol) in anhydrous methanol (2*a*) and in 50 vol. % system containing 0.01 M Tris—HCl, pH = 8.2, and methanol (2*b*).


1 h
3 h
4 h
8 h
24 h
a


1 h
2 h
4 h
6 h
24 h
48 h
b

tion products could be observed already in the first hours of the reaction course. If the substrate was incubated at the same conditions only in anhydrous methanol without trypsin and arginine, no deacetylation could be observed even after 80 h of incubation.

Table 1

Relative activity of trypsin after the reaction of deacetylation of the peracetylated β-D-xylopyranoside at 48 °C

Substrate	Reaction time/h	Relative activity/%[c]
I[a]	68	3.7
II[a]	80	6.8
II + arginine[a]	80	6.5
I[b]	72	46

a) Reaction in anhydrous methanol; *b*) in isooctane; *c*) relative activity = specific activity of trypsin prior to reaction/specific activity of trypsin after the reaction × 100.

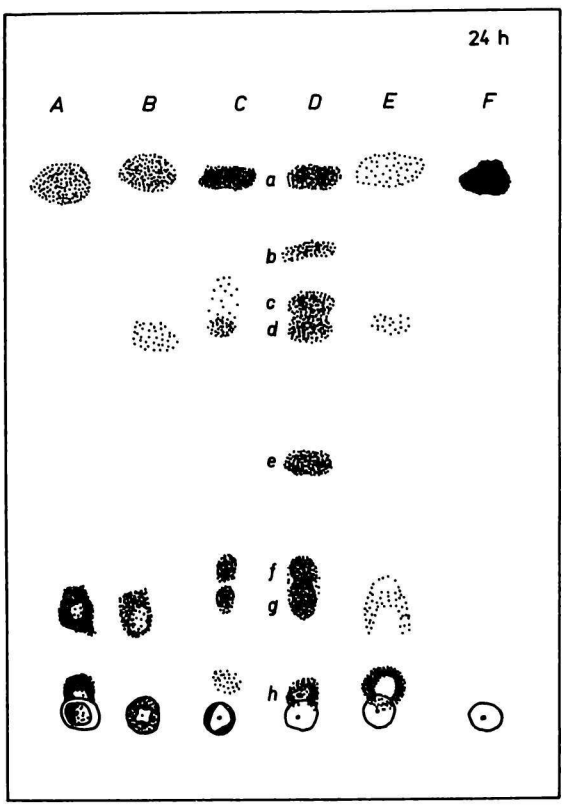


Fig. 3. Deacetylation of methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside at 48 °C in anhydrous methanol after 24 h incubation, catalyzed by serine hydrolases (á 10 mg): *A.* Lipase; *B.* chymotrypsin; *C.* trypsin; *D.* standard mixture consisting of the substrate and reaction products with different acetylation degree. Sequence and denotation is as in Fig. 1; *E.* plasmatic pseudocholinesterase; *F.* blank incubation without enzyme.

Influence of the reaction medium and reaction conditions on the enzymic activity of trypsin is evident from Table 1. When methyl 2,3,4-tri-*O*-acetyl-β-D--xylopyranoside (*I*) was used as a substrate, greater decrease of the specific activity was observed than in the case when more hydrophobic umbelliferyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (*II*) was used. Addition of arginine did not affect specific activity of trypsin in the course of the reaction. However, when the enzyme was incubated with the substrate in the substantially more hydrophobic medium of isooctane at the same conditions, almost 46 % of initial specific enzymic activity was retained (deacetylation, however, did not take place in this medium). This fact is in good accordance with the observations of *Klibanov et al.* [6] that increase of the hydrophobicity of the medium and decrease of water content in it leads to higher thermostability of the enzyme.

Data on the effect of the serine hydrolases on the peracetylated saccharides in pure anhydrous media lack as yet in the literature. That is why we studied the process of the deacetylation of methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside in anhydrous methanol in the presence of serine hydrolases trypsin, chymotrypsin, lipase, and pseudocholinesterase. As seen from Fig. 3, the reaction proceeds although not selectively. The reaction mixture contains only minimal amount of di-*O*-acetylated derivatives of methyl β-D-xylopyranoside. Major portion of the original substrate undergoes deacetylation up to the monosubstituted derivatives or even free methyl β-D-xylopyranoside. Addition of the acceptors causes slight increase of the reaction rate, but even in this case selectivity of the deacetylation does not increase. Deacetylation without the acceptors, only in methanol in the presence of the enzymes that were not treated as described in Experimental, does not occur at all at the same experimental conditions.

Serine hydrolases, hence, are able to catalyze deacetylation of the peracetylated xylosides in hydrophilic medium — anhydrous methanol, although none of the stereospecifically different positions is privileged. Thermostability of the enzyme is relatively low in this medium.

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