Carbohydrate Research, 194 (1989) 321–324
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Substrate specificity of naringinase, an α-L-rhamnosidase from *Penicillium decumbens**

FRANCIS MICHON, VINCE POZSGAY, JEAN-ROBERT BRISSON, AND HAROLD J. JENNINGS
Division of Biological Sciences, National Research Council of Canada, Ottawa, Ont. K1A OR6 (Canada)
(Received November 7th, 1988; accepted for publication May 1st, 1989)

L-Rhamnose is a constituent of many important bacterial and plant polysaccharides[1–3], glycosides[4], and other glycoconjugates[5,6]. Determination of the anomeric configuration of L-rhamnopyranosides can best be accomplished by ^{13}C-n.m.r. spectroscopy[7], based on the stereochemical dependence of the C-1–H-1 one-bond coupling constant[8,9]. The inherent insensitivity of this technique requires, however, relatively large amounts of sample and/or long acquisition times.

We have recently described the successful use of naringinase, a commercial preparation obtained from *Penicillium decumbens* containing both α-L-rhamnosidase (E.C. 3.2.1.40) and β-D-glucosidase (E.C. 3.2.1.21) activities, in the structure determination of the complex group-specific polysaccharide of group B *streptococcus*[10,11]. In fact the results of these experiments could also have been used to confirm the configuration of some of its rhamnopyranosyl residues if the specificity of the rhamnosidase had been known with certainty at this time. Several α-L-rhamnosidases, isolated from various bacterial strains, have been described and the substrate specificity of that obtained from *Aspergillus niger* was investigated in detail[12,13]. We now report on the substrate specificity of naringinase, using a number of synthetic mono- and oligo-rhamnoside substrates as shown in Table I, which also lists the products of hydrolysis. The optimal hydrolytic conditions (temperature, pH, ionic strength of the buffer) were chosen from previous work[12]. The structures of the product of hydrolysis were established by ^1H-n.m.r. spectroscopy at 500 MHz.

Of all the possible methyl rhamnopyranosides (**1–4**), only methyl α-L-rhamnopyranoside was a substrate for naringinase, indicating that the rhamnosidase activity of this enzyme is specific for α-L-rhamnopyranosyl linkages (Table I). In contrast, methyl 1-thio-α-L-rhamnopyranoside (**5**) was not hydrolyzed despite the fact that it has the required configuration. However, 1-thioglycosides are

*This is National Research Council of Canada Publication No. 30757.

0008-6215/89/$ 03.50

TABLE I

SUBSTRATE SPECIFICITY OF NARINGINASE FROM *Penicillium decumbens*

Substrates[a]	*Products*[b]
1 α-L-Rha*p*-OMe[15]	→L-Rha[c]
2 β-L-Rha*p*-OMe[16]	ND
3 α-D-Rha*p*-OMe[17]	ND
4 β-D-Rha*p*-OMe[d]	ND
5 α-L-Rha*p*-SMe[18]	ND
6 α-L-Man*p*-OMe[19]	→L-Man[c]
7 α-D-Man*p*-OMe[19]	ND
8 α-L-Rha*p*-(1→2)-α-L-Rha*p*-OMe[20]	α-Rha + (**1**) → L-Rha
9 α-L-Rha*p*-(1→3)-α-L-Rha*p*-OMe[20]	L-Rha + (**1**) → L-Rha
10 α-L-Rha*p*-(1→4)-α-L-Rha*p*-OMe[21]	L-Rha + (**1**) → L-Rha
11 α-L-Man*p*-(1→2)-α-L-Rha*p*-OMe[18]	L-Man + (**1**) → L-Man + L-Rha
12 α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*OMe[20]	L-Rha + (**1**) → L-Rha
13 α-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→2)-α-L-Rha*p*OMe[22]	L-Rha + (**1**) → L-Rha
14 α-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→4)-α-L-Rha*p*OMe[22]	L-Rha + (**1**) → L-Rha
15 α-L-Man*p*-(1→4)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-OMe[18]	L-Man + L-Rha + (**1**) → L-Man + L-Rha

[a]For compounds **1**–**7** 10 mU of naringinase was used in the experiment, for **8** and **9** 20 mU, and for **12**–**15** 40 mU. [b]ND, substrate not degraded. [c]Under the conditions used, product appeared after an hour of incubation, but the reaction did not go to completion, probably because of inhibition of the enzyme by the product. The same was true for the hydrolysis of **8**–**15**, where **1** was an intermediate product. [d]Methyl β-D-rhamnopyranoside was obtained by acid-catalyzed anomerization of **3** followed by chromatography[15]. [e]Product appeared in the reaction mixture, after 10 h of incubation.

frequently inhibitors of glycoside-cleaving enzymes[13] and indeed this property was confirmed for methyl 1-thio-α-L-rhamnopyranoside in a competitive assay (see Experimental), which showed a *ca.* 30% reduction in the degree of hydrolysis of **1** in the presence of a tenfold molar excess of **5**. Methyl α-L-mannopyranoside (**6**) was very slowly hydrolyzed to L-mannose, indicating that the enzyme tolerates the replacement of the methyl group at C-5 by a hydroxymethyl group, although the substitution significantly retards the rate of hydrolysis of this simple glycoside. As expected, methyl α-D-mannopyranoside (**7**) was not hydrolyzed at all. The substrate specificity of the naringinase α-L-rhamnosidase differs from that of the rhamnosidase isolated from *Aspergillus niger,* which was found inactive towards α-L-mannosides[14].

Disaccharide **8**, containing an α-(1→2) rhamnopyranosyl linkage, was rapidly hydrolyzed by naringinase to yield L-rhamnose and methyl α-L-rhamnopyranoside (Table I), the latter being slowly hydrolyzed to L-rhamnose. The 1→3-(**9**) and 1→4-linked (**10**) rhamnose disaccharides were hydrolyzed in a similar fashion, indicating that the linkage site in rhamnobioses is not a crucial factor in determining the enzyme specificity.

This feature of the specificity was confirmed by treating rhamnose trisaccharides **12, 13**, and **14** with naringinase. Trisaccharide **12**, which contains two sequential α-L-(1→2) linkages, was also rapidly hydrolyzed to L-rhamnose and methyl α-L-rhamnopyranoside (**1**), the latter product again being slowly hydrolyzed to L-rhamnose (Table I). A similar hydrolytic pattern was also exhibited by trisaccharides **13** and **14**, the former containing sequential α-L-(1→2) and α-L-(1→4) linkages, and the latter containing two sequential α-L-(1→4) linkages.

Surprisingly, in contrast to methyl α-L-mannopyranoside (**6**), which was hydrolyzed slowly compared to methyl α-L-rhamnopyranoside (**1**), disaccharide **11** and trisaccharide **15**, both having terminal α-L-mannopyranosyl residues, were as effective as substrates for naringinase as their respective analogs (**8** and **12**) having nonreducing terminal α-L-rhamnopyranosyl residues. Thus the hydroxymethyl group at C-6 of their terminal residues does not significantly retard the hydrolysis of these oligomeric molecules. This may indicate that the α-L-rhamnosidase component of naringinase is primarily an exoglyconase rather than a glycosidase. In any event the foregoing observations show that naringinase specifically cleaves nonreducing terminal α-L-rhamnopyranosyl linkages in rhamnose di- and trisaccharides, independently of the point of attachment of their interglycosidic linkages. Some corresponding α-L-mannopyranosyl residues are similarly cleaved.

EXPERIMENTAL

Naringinase from *Penicillium decumbens* was purchased from Sigma Chemical Co, lot no. 21F-84201. The preparation contained 400 U/g of α-L-rhamnosidase and 5 U/g of β-D-glucosidase activity. ^{1}H-n.m.r. spectra were run on a Bruker AM 500 spectrometer in 5 mm tubes containing acetone as an internal

standard (δ = 2.225 p.p.m.). Samples (0.5 mL) were run at 320 K in D_2O containing the appropriate substrate. Buffer chemicals, substrates, and enzymes were dissolved in D_2O, then freeze-dried twice prior to use in order to remove all exchangeable protons. The individual experiments were set up as follows. Prior to enzymatic digestion an FID was recorded for each substrate (5.0 mg) in 0.4 mL of buffer (100mM sodium acetate, pH 4.0), the enzyme (from 10 to 40 mU depending on the substrate) in 0.1 mL of the same buffer was then added, and FID's were recorded for 2 h at regular intervals (every min or every 30 s) during the course of hydrolysis. Samples of compounds **1–7**, whose rates of hydrolysis were very slow, were further incubated with the enzyme for an additional 16 h and the course of the hydrolyses was monitored by ^{1}H-n.m.r. at 320 K. Hydrolysis was indicated by the appearance of signals for H-1α and H-1β of L-rhamnose at 5.112 p.p.m. and 4.864 p.p.m. respectively. The corresponding signals for L-mannose were found at 5.180 and 4.896 p.p.m., respectively. For the inhibition experiment, methyl α-L-rhamnopyranoside was incubated for 16 h together with 10 molar equivalents of methyl 1-thio-α-L-rhamnopyranoside and naringinase in the buffer as described above.

ACKNOWLEDGMENT

The authors thank Professor András Lipták (Debrecen, Hungary) for his generous gift of compounds **13** and **14**.

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