0273-2289/97/6201—0061$10.25

Reverse Hydrolysis Reaction in Aqueous Medium Without Any Cosolvent

Application to Synthesis of Glycosidic Esters of Tyrosine

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Received January 22, 1996; Accepted March 14, 1996

ABSTRACT

The synthesis of L-tyrosine fructosyl ester, from fructose and L-tyrosine methyl ester, was carried out by a transesterification reaction catalyzed by α-chymotrypsin in water without cosolvent. The effect of fructose concentration and temperature for the transesterification reaction were determined on both specific activities and product yield. The influence of the presence of fructose has been studied regarding α-chymotrypsin and L-tyrosine fructosyl ester stabilities. It appeared that an increase of temperature enhanced enzyme activity but slumped the product yield because of the very weak stability of tyrosine fructosyl ester.

Index Entries: α-Chymotrypsin; transesterification; aqueous medium; glycosidic esters; tyrosine-fructosyl-ester.

INTRODUCTION

Amino acids derivatives have important applications in food and pharmacological areas. In the cosmetic industry, synthesis of hydrophilic tyrosine derivatives has denoted interest *(1,2)* in order to increase the solubility of tyrosine (melanin precursor) in water. *N*-L-malyl-tyrosine, a highly water

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soluble product, is already applied in skin tanning and skin protection cosmetics.

Saccharide-amino acid conjugates synthesis has already been described in aqueous medium: It consisted in a transfer of a sugar on an amino acid as acceptor *(3,4)* using glycosidases. In these cases, product yields rarely exceeded 10%. Moreover, it has been demonstrated that serine protease like subtilisin could transfer amino acids like phenylalanine, methionine, or alanine on di- and oligosaccharides or polyols *(5)*. Such reactions were possible in anhydrous dimethylformamide with high concentrations of enzyme that did not allow an industrial application.

Proteases were also used to acylate sugar with fatty acids in organic solvents *(6–9)*. In the presence of suitable anhydrous solvent (e.g., pyridine, DMF, and so on) they were capable of regioselectively acylating sugars. The acylation using proteases, in pure water with high concentrations of fructose, is close to synthesis in water and hydrophilic organic solvents medium, such as ethanol or polyols, which has been extensively reported *(10,11)*. A protective effect on enzymatic thermostability *(12,13)*, and shift of reaction equilibria *(14)* by diminution of water content and water activity was observed in these cases.

The present study concerns synthesis of glycosidic esters of tyrosine from L-tyrosine methyl ester and fructose in water without any cosolvent. Effects of fructose content and temperature that are of primary interest with respect to the hydrolytic and transesterification activities of α-chymotrypsin and product yield have been carefully checked.

MATERIALS AND METHODS

Materials

α-Chymotrypsin (EC 3.4.21.1) Type II from bovine pancreas and L-tyrosine methyl ester hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MD). Extra pure D-fructose was obtained from Merck (Mannheim, Germany).

Hydrolysis/Transesterification Reactions

All concentrations were expressed in mass ratio and the total mass was 12 g in all cases. 0.6 g (5% w/w) of tyrosine methyl ester hydrochloride (Tme) and various amounts of fructose were dissolved in 0.2*M* phosphate buffer pH 6.5 (the reaction medium mass was adjusted to 11.9 g with buffer). Then, the reaction was started by addition of 100 mg of a 500 μg/g α-chymotrypsin solution in buffer. Aliquots of 100 μL were extracted at several time intervals from the reaction mixture, were mixed with 150 μL of 30% (w/w) trichloroacetic acid, in order to stop the reaction, and stored at 4°C until analysis.

HPLC Analysis

Substrates and products concentrations were analyzed by reverse phase HPLC using a Nucleosil C18 column (250 × 4.6 mm, 5 μm, 100 Å) coupled with a UV spectrophotometer (Lambda 1000 ICS) at 274 nm and thermostated at 50°C with a WATERS oven. Samples were eluted isocratically with water: acetonitrile: acetic acid (89:10:1, v/v/v) containing 5 *mM* 1-hexane sulfonic acid (Sigma) at 1 mL/min flow rate. One unit of activity was defined as the amount of enzyme that produced 1 μmole of the hydrolysis (L-tyrosine) or transesterification (L-tyrosine fructosyl ester) product per minute. Product yield was expressed as the ratio of tyrosine fructosyl ester at its maximum concentration on initial tyrosine methyl ester.

^{13}C-NMR and ^{1}H-NMR Measurements

^{13}C-NMR spectra was recorded on a Brucker AC-300 spectrometer (75.43 MHz). ^{1}H-NMR spectra was recorded on a Brucker 400 MHz. The chemical shifts were determined at 25°C and the sample (50 mg/mL) was dissolved in deuterium oxide.

Study of Thermal Stability with and Without Fructose

N-acetyl-L-tyrosine ethyl ester (ATEE, Sigma) was used as standard substrate. All remaining reagents were of analytical grade. The esterase activity of α-chymotrypsin was determined by the pH-stat method described in detail by Wilcox *(15)* using a Metrohm pH-stat (Herisau), equipped with a pH-meter (mod. 691), an autoburette (Dosimat mod. 665 and Impulsomat mod. 614).

The reaction mixture was as follows: a 10 mL sample of 50 m*M* ATEE in 5 m*M* Tris-HCl buffer pH 7.0 containing ethanol (30% v/v) and 20 m*M* $CaCl_2$, was placed into a thermostated (30°C) reaction vessel. The reaction was started by addition of 100 μL of α-chymotrypsin solution in 50 m*M* HCl (in order to avoid autolysis phenomenon). The pH was maintained constant at a value of 7.0 by continuous addition of 10 m*M* NaOH. Aqueous solutions of α-chymotrypsin were prepared with and without fructose (30 and 50% (w/w) fructose solutions) at a concentration of 250 μg/g of storage medium with 200 m*M* phosphate buffer pH 6.5. Each mixture was incubated at 40, 50, and 60°C. At constant time intervals, aliquots of 0.5 mL were extracted from the incubation tube and added to 2 mL of 50 m*M* HCl. The residual activity was determined as described in the HPLC Analysis section.

RESULTS AND DISCUSSION

In protease-catalyzed ester synthesis, enzymatic activity and product yield are highly dependent on the water content in the reaction medium. A high water concentration will negatively affect transesterification and enhance hydrolysis. Frequently, the addition of water miscible or immiscible organic solvent is used to decrease the water content and water activity in order to shift the equilibrium towards synthesis.

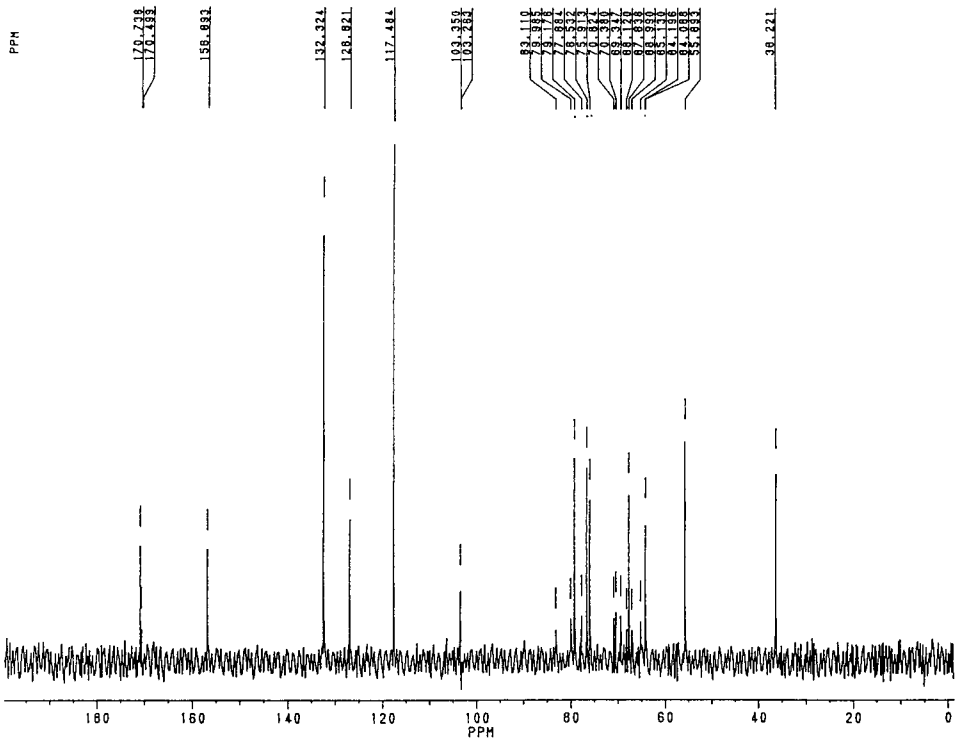


Fig. 1. ^{13}C-NMR spectrum of L-tyrosine fructosyl ester.

Our reaction was carried out in aqueous buffer without any cosolvent, with L-tyrosine methyl ester as acyl donor and D-fructose as acyl acceptor. The synthesis of L-tyrosine fructosyl ester has been demonstrated by ^{13}C-NMR (Fig. 1) and ^{1}H-NMR identification. The spectra, in comparison with spectra of pure fructose, exhibit the presence of various isomers and impurities, and only 6-O-L-tyrosyl-β-D-fructofuranose has been clearly identified (Table 1) as the main product with a 95% purity.

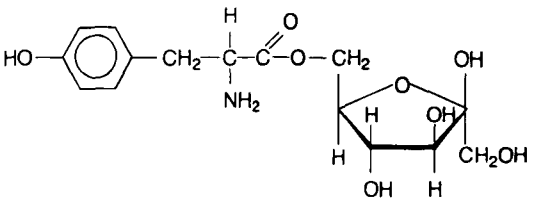
Effect of Fructose Content

Although maximum synthesis generally takes place at pH values from 9 to 11 *(16)*, a low acidic pH has been chosen because glycosidic ester are unstable in alkaline pH because of sugar ring opening under basic conditions. Moreover, Oka and Morihara *(17)* have demonstrated that a neutral pH was more efficient in the case of synthesis in aqueous buffer and when the amine function of tyrosine methyl ester was unprotected. Indeed an alkaline pH will enhance the synthesis of the dipeptide Tyr-Tyr.

Figure 2 presents the effect of fructose concentration on both the hydrolytic activity and the transesterification activity of α-chymotrypsin at 40°C. This figure indicates that the hydrolytic activity decreases proportionally to the increase in fructose concentration. Furthermore, the transesterification activity profile is a bell-shaped curve with a maximum of

Table 1
Chemical Shifts (ppm) and Structure of 6-O-L-Tyrosyl-β-D-Fructofuranose

Carbon	6-O-L-tyrosyl-β-D-fructofuranose in D_2O
C=O	170.7
C_4 (phenyl)	156.7
C_2 and C_6 (phenyl)	132.3
C_1 (phenyl)	126.8
C_3 and C_5 (phenyl)	117.5
C_2 (fructose)	103.3
C_5 (fructose)	79.2
C_3 (fructose)	76.5
C_4 (fructose)	75.9
C_6 (fructose)	67.9
C_1 (fructose)	64.1
-CH-NH_2	55.7
phenyl-CH_2-	36.2



enzyme activity at a 30% (w/w) fructose concentration. Between 30% and 50% (w/w) the synthesis activity slightly decreases and then slumps with higher fructose concentration. This last phenomenon can be attributed to the rise in the viscosity of the reaction media.

Figure 3 represents the effect of fructose concentration on the product yield. It is noteworthy that the maximum of transesterification is reached at 50% (w/w) fructose concentration. This curve is close to the plot of the ratio: transesterification activity upon hydrolytic activity against fructose concentration indicating the preferential pathway. The ratio is maximum with 50% (w/w) fructose, and the reaction is more and more directed towards transesterification.

In such a medium, the transesterification product is fully soluble whereas L-tyrosine precipitates at low concentration. It is well known that the reaction yield increases with decreasing solubility of the product, thus the precipitation of L-tyrosine will shift the equilibrium point in favor of the hydrolysis. Moreover, because of its high solubility, L-tyrosine fructosyl ester is not prevented from hydrolysis. Consequently, it is, therefore, of the utmost importance to stop the reaction at the kinetic optimum.

Temperature Effect

Figure 4 shows both hydrolytic and transesterification activities profiles as functions of temperature in presence of two fructose concentrations

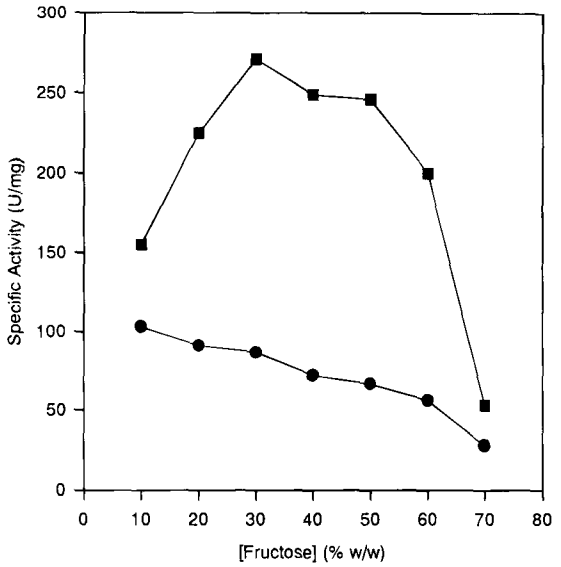


Fig. 2. Influence of fructose concentration on both the hydrolytic (●) and transesterification (■) activities of α-chymotrypsin using L-tyrosine methyl ester as substrate at 40°C.

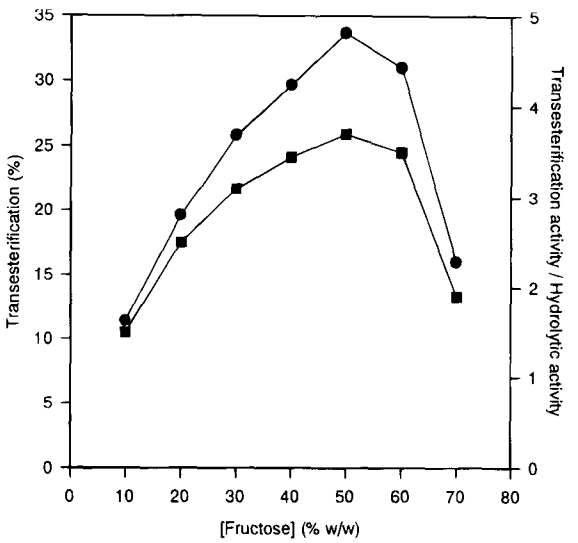


Fig. 3. Influence of fructose concentration on both the transesterification product yield (●) and the ratio: transesterification activity upon hydrolytic activity (■).

[30% and 50% (w/w)]. In both cases, hydrolytic activity increases with temperature. This increase is essentially a result of the thermal hydrolysis of L-tyrosine fructosyl ester (Table 2) and at a lesser degree to L-tyrosine methyl ester one.

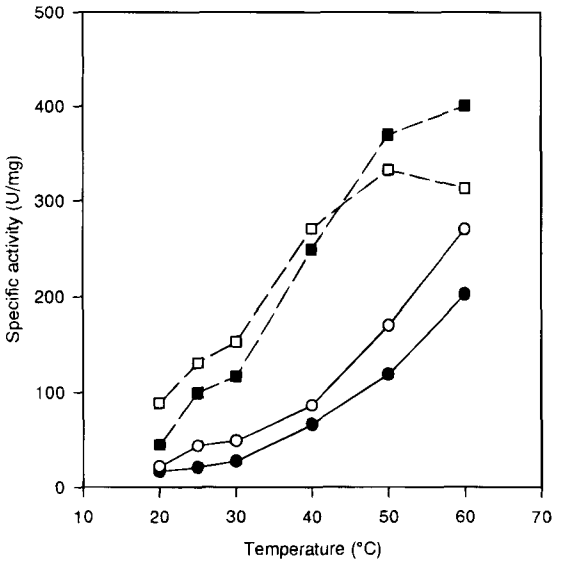


Fig. 4. Influence of temperature on both the hydrolytic (● and ○) and transesterification (■ and □) activities of α-chymotrypsin using L-tyrosine methyl ester as substrate at 40°C with 30% (w/w) fructose (hollow symbols) and 50% (w/w) fructose (filled symbols).

Table 2
Effect of Temperature on the Half-Life Constant of L-Tyrosine Fructosyl Ester in 0.2*M* Phosphate Buffer pH 6.5 with 30% and 50% Fructose

Temperature	30% fructose	50% fructose
40°C	56 min	81 min
50°C	19 min	30 min
60°C	4 min	8 min

With respect to transesterification activity, temperature enhances activity too. Up to 40°C, activity with 30% (w/w) fructose is already above activity with 50% (w/w) fructose. With higher temperature, activity with 50% (w/w) fructose becomes superior. This inversion is a result of both enzyme (Table 3) and product (Table 2) thermal denaturation. A protective effect on both enzyme and product is observed and this effect is more marked with high concentration of fructose.

These phenomena are similar to those observed with polyols. Indeed polyols have a stabilizing effect on α-chymotrypsin thermal stability proportionally to their concentration *(13)*. Like polyols, fructose has simultaneously a double role. It may act as a nucleophile acceptor of the acyl intermediate and as a water content reducing agent. Fructose is a high hygroscopic sugar and hydrogen bonds formed with fructose should play an important role in enzyme stabilization by increasing the

Table 3
Effect of Temperature on the Half-Life Constant of α-Chymotrypsin with and without Fructose in 0.2*M* Phosphate Buffer pH 6.5. The Protective Effect (Determined with Half-Life Constant of α-Chymotrypsin in Buffer only as Reference) is Noted in Parentheses

Temperature	buffer only	30% fructose	50% fructose
40°C	2160 min	3000 min (1.4)	10080 min (4.7)
50°C	78 min	300 min (3.8)	1800 min (23)
60°C	0.8 min	5 min (6.2)	40 min (48)

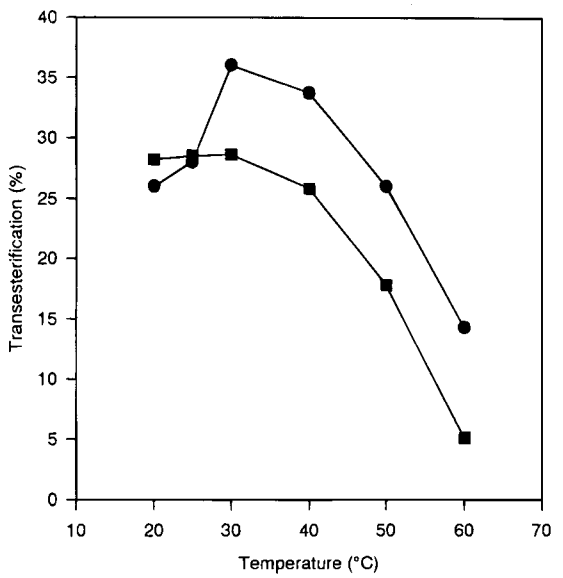


Fig. 5. Influence of temperature on transesterification product yield with 30% (w/w) fructose (■) and 50% (w/w) fructose (●).

degree of organization of water molecules and changing microenvironment of the enzyme by 'immobilization' of the water medium *(13)*. The influence of temperature on the reaction yield was also studied (Fig. 5). Although a temperature rise enhanced transesterification activity, temperatures above 40°C greatly decreased the product yield. At high temperature, the thermal hydrolysis of L-tyrosine fructosyl ester slumps the yield and when the temperature reaches 60°C, enzyme deactivation balances the activity enhanced by temperature. For temperatures below 30°C, the viscosity goes up and product yield with 50% (w/w) fructose falls and is lower than yield with 30% (w/w) fructose which is leveling off at 28.5%.

The shape of this curve will be largely influenced by enzyme concentration. Indeed, with high enzyme concentration the maximum of synthesis will be reached earlier. Thus the thermal hydrolysis of L-tyrosine

fructosyl ester will have less influence and it will be possible to obtain maximum product yield at a higher temperature with a more important enzyme concentration.

CONCLUSION

The synthesis of L-tyrosine fructosyl ester in aqueous buffer has been demonstrated. The maximal activity level was obtained for 30% (w/w) fructose concentration and maximum yield (34%) for 50% (w/w) fructose at 40°C. The yield could be easily increased with higher enzyme concentration in order to balance the thermal hydrolysis of product by reducing time of maximum synthesis. Finally, the protective effect of fructose on both enzyme and L-tyrosine fructosyl ester which is highly sensitive to rise of temperature has been displayed.

This model transesterification in water-sugar medium without any cosolvent allows to consider synthesis of various saccharide-amino acid compounds playing on sugar and substrate specifity of proteases.

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