ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 10 (2000) 565–570

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

N-acetyl-L-arginine ethyl ester synthesis catalysed by bovine trypsin in organic media

L.M. Simon *, M. Kotormán, K. Maráczi, K. László

Department of Biochemistry, József Attila University, P.O. Box 533, Szeged H-6701, Hungary

Received 13 July 1999; received in revised form 22 February 2000; accepted 22 February 2000

Abstract

The bovine trypsin-catalysed synthesis of *N*-acetyl-L-arginine ethyl ester from *N*-acetyl-L-arginine and ethanol was studied in various organic solvents (dimethyl sulfoxide, dioxane, dimethylformamide, acetonitrile, acetone, tetrahydrofuran, chloroform, toluene, carbon tetrachloride, cyclohexane and *n*-hexane). The highest yield was achieved in acetonitrile after incubation for 6 or 24 h. The optimal conditions for ester synthesis in acetonitrile for 6 h were as follows: 5.0 mM *N*-acetyl-L-arginine, 10.0 M ethanol, 7.2 mg trypsin, 2.87% water, total volume 10.3 ml, pH 7.0 and 30°C. The hydrolytic activity of trypsin was determined after incubation for 6 days, when 87.7% of the original activity remained, suggesting that acetonitrile caused little inactivation of the enzyme. The synthetic reaction resulted in a maximal 79.3% conversion under optimized conditions after incubation for 48 h. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Bovine trypsin; *N*-acetyl-L-arginine ethyl ester synthesis; Optimization; Acetonitrile

1. Introduction

The use of organic solvents in enzymatic syntheses has recently attracted considerable attention. There are many advantages of enzymatic syntheses in organic media, such as the increased solubility of hydrophobic substrates, with shifts in the thermodynamic equilibria in the synthetic direction, and the ease of both product and enzyme recovery. Attempts have been made to establish rules relating to the choice of a suitable solvent for each type of reaction. In general, hydrophobic solvents are preferred, because the retention of enzyme activity is favourable in these solvents. However, in many esterification reactions, polar substrates are used. The solvent influences the activities and/or stabilities of the enzymes and also the equilibrium position of the reaction [1,2].

Peptide syntheses catalysed by proteases have been extensively studied as an alternative to the chemical synthesis of polypeptides, because they generally do not require special activation of the carboxyl group, they are highly specific, and there is no need for protecting groups [3,4]. Ester synthesis and transesterification with pro-

* Corresponding author. Tel.: +36-62-454-105; fax: +36-62-432-487.

E-mail address: simon@biocom.bio.u-szeged.hu (L.M. Simon).

1381-1177/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S1381-1177(00)00098-9

teases have been less widely studied. Shih et al. [5] described the esterification of *N*-Cbz-L-amino acids with secondary alcohols by Celite-immobilized protease and lipase. Alkylated trypsin has been applied for the esterification of D-glucose with oleic acid [6]. The equilibrium and kinetics of *N*-acetyl-L-tryptophan phenylethyl ester synthesis with agarose chymotrypsin were studied by Blanco et al. [7] in organic media.

The present work reports the effects of solvents on the esterification of *N*-acetyl-L-arginine with different aliphatic alcohols. The ethyl ester was produced in the highest amount, and the reaction conditions (ratio of substrates, water content, appropriate temperature and pH) were examined in detail. *N*-Substituted amino acid esters serve as substrates of proteases in hydrolytic reactions and can be used in peptide syntheses to eliminate water production in organic media.

2. Materials and methods

2.1. Materials

Bovine pancreas trypsin (EC 3.4.21.4), *N*-acetyl-L-arginine, *N*-acetyl-L-lysine and *N*-benzoyl-L-arginine ethyl ester were purchased from Sigma-Aldrich (Budapest). The specific activity of the trypsin was 15,900 units/mg. All other chemicals were reagent-grade products (Reanal, Budapest).

2.2. *N*-acetyl-L-arginine ester synthesis and analysis

The standard reaction mixture consisted of one or other above-listed *N*-protected-L-amino acids (5 mM), 10.0 M alcohol, 7.2 mg trypsin dissolved in 20 μl 1 mM HCl, 125 μl 0.1 M triethanolamine/HCl buffer (pH 7.0) and 4 ml organic solvent (mainly acetonitrile) in a total volume of 10.3 ml. The reaction was started by the addition of trypsin and the mixture was stirred at 450 rpm for 6 or 24 h at 30°C After appropriate incubation periods, samples were withdrawn and analysed for *N*-acetyl-L-amino acid and hydrolytic activity. The water content in the reaction mixture was determined by Karl–Fischer titration [8].

The amount of *N*-acetyl-L-arginine ester was determined indirectly from the amount of *N*-acetyl-L-arginine remaining. The *N*-acetyl-L-arginine measurements were performed at pH 9.5 by pH stat titration with an automatic titrator (Radiometer, Copenhagen, Denmark). The titrant used was 10 mM NaOH. The titration method was validated by titrating mixtures of 5 mM of *N*-acetyl-L-arginine and different amounts (0.5, 1 and 3 mM) of its ethyl ester. The consumption of alkali was not influenced by the presence of the ester.

2.3. Assay of ester hydrolase activity

The activity of trypsin was measured by following the increase in absorbance at 253 nm [9]. The reaction mixture (3 ml) contained 0.9 mM *N*-benzoyl-L-arginine ethyl ester, 46.7 mM Tris/HCl buffer (pH 8.0) and a 100 μl aliquot of enzyme. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 μmol *N*-benzoyl-L-arginine ethyl ester per minute under the assay conditions.

3. Results and discussion

Trypsin-catalysed peptide syntheses have been thoroughly studied during recent decades because of the great importance of the different biologically active peptides, whereas little attention has been paid to esterification and transesterification reactions. Investigation of the direct esterification of *N*-protected amino acids offers a possibility to compare the behaviour of trypsin in organic solvents in the presence of different

substrates, and additionally provides an insight into the active site of the enzyme. The different *N*-protected amino acid esters can be used as substrates of proteolytic enzymes in the esterase reaction.

3.1. Synthesis of N-acetyl-L-arginine esters

In the esterification of *N*-acetyl-L-arginine, methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol and pentan-1-ol were used at 10.0 M in acetonitrile. The results are summarized in Table 1. The highest yield of *N*-acetyl-L-arginine ester was achieved with ethanol during reaction for 6 or 24 h. With increase in the number of carbon atoms in the alcohol, the yield of esterification decreased. This might be due to the changes in the lipophilicity of the different alcohols. In the further experiments, the synthesis of *N*-acetyl-L-arginine ethyl ester was studied.

The transesterification of lysine esters (methyl, *n*-propyl and *n*-butyl) with ethanol catalysed by bovine trypsin resulted in 74–80% ethyl ester at pH 6.2 and at 25°C for 50 h in 95% ethanol [10].

3.2. Effects of organic solvents

The synthesis of *N*-acetyl-L-arginine ethyl ester was examined in the following solvents: dimethyl sulfoxide, 1,4-dioxane, *N*,*N*-dimethylformamide, acetonitrile, acetone, tetrahydrofuran, chloroform, toluene, carbon tetrachloride, cyclohexane and *n*-hexane. The amounts of *N*-acetyl-L-arginine ethyl ester formed during incubation for 6 and 24 h are presented in Table 2. No ester synthesis occurred in *N*,*N*-dimethylformamide or dimethyl sulfoxide. The yield of ester in the different solvents did not exhibit any correlation with the log *P* values of the solvents. Acetonitrile proved the best solvent for the synthesis of *N*-acetyl-L-arginine ethyl ester. For the further experiments, acetonitrile was chosen as solvent in the esterification reactions. Sugar esterification with modified trypsin was performed in dimethylformamide [6], but the free trypsin-catalysed peptide synthesis was studied in acetonitrile [4].

Table 1
Yields of different *N*-acetyl-L-arginine ester syntheses
The syntheses were carried out in a reaction mixture containing 5.0 mM *N*-acetyl-L-arginine, 10.0 M alcohol, 7.2 mg trypsin, 125 μl triethanolamine/HCl buffer (pH 7.0) and 4.0 ml acetonitrile at a water content of 2.87% and at 30°C.

Alcohol	Yield (%)	
	6 h	24 h
Methanol	4.0	14.6
Ethanol	50.9	73.6
Propan-1-ol	13.9	54.2
Propan-2-ol	7.4	30.3
Butan-1-ol	11.7	46.0
Pentan-1-ol	7.9	33.4

Table 2
N-acetyl-L-arginine ethyl ester synthesis in different organic solvents
The reaction mixture contained 5.0 mM *N*-acetyl-L-arginine, 10.0 M ethanol, 125 μl 0.1 M triethanolamine/HCl buffer (pH 8.0), 2.0 mg trypsin and 4 ml organic solvent.

Solvent	Yield (%)	
	6 h	24 h
Dimethyl sulfoxide	0.0	0.0
1,4-Dioxane	14.4	15.6
Dimethylformamide	0.0	0.0
Acetonitrile	6.1	25.9
Acetone	2.9	14.9
Tetrahydrofuran	0.6	3.2
Chloroform	0.4	2.4
Toluene	4.0	22.4
Carbon tetrachloride	0.7	5.6
Cyclohexane	3.2	9.7
n-Hexane	5.4	21.5

3.3. Synthesis of N-protected amino acid ethyl esters

Since the specificity of trypsin is known for both L-arginine and L-lysine, the ester syntheses with *N*-acetyl-L-arginine and with *N*-acetyl-L-lysine were compared. The chymotryptic activities of trypsin with *N*-acetyl-L-tyrosine and *N*-benzoyl-L-phenylalanine were also studied. The esterification results are listed in Table 3. The yield of *N*-acetyl-L-arginine ethyl ester was the

Table 3
Yields of different *N*-protected-L-amino acid ethyl ester syntheses The reaction mixture contained 5.0 mM *N*-protected-L-amino acid, 10.0 M ethanol, 7.2 mg trypsin, 125 μl 0.1 M triethanolamine/HCl buffer (pH 7.0), and 4 ml acetonitrile. The synthesis was performed at 30°C and at 2.87% water content.

Amino acid	Yield (%)	
	6 h	24 h
N-acetyl-L-lysine	30.7	49.0
N-acetyl-L-arginine	50.9	73.6
N-acetyl-L-tyrosine	12.6	14.2
N-benzoyl-L-phenylalanine	0.0	0.0

highest on incubation for both 6 and 24 h (50.89% and 73.58%). *N*-acetyl-L-tyrosine ethyl ester synthesis from *N*-acetyl-L-tyrosine and ethanol catalysed by immobilized chymotrypsin at pH 4.2 and 25°C in an aqueous chloroform biphasic mixture for 30 h resulted in an 85–90% ester yield [11,12].

3.4. Effects of substrate concentration

The synthesis of *N*-acetyl-L-arginine ethyl ester was studied in the *N*-acetyl-L-arginine concentration range 1.8–9.0 mM at pH 7.0 and 30°C. To achieve the highest yield of *N*-acetyl-L-arginine ethyl ester, approximately 5.0 mM *N*-acetyl-L-arginine was necessary in the reaction mixture.

3.5. Effects of ethanol

The effects of the substrate concentration on the synthesis of *N*-acetyl-L-arginine ethyl ester with trypsin were studied at ethanol concentrations between 6.6 and 16.7 M in acetonitrile. As can be seen from Fig. 1, approximately 10.0 M ethanol was needed for the highest conversion.

3.6. Effects of enzyme concentration

In these experiments, the amount of trypsin in the reaction mixture was varied between 1 and 8 mg. The maximal conversion of *N*-acetyl-L-arginine ethyl ester was achieved with the use of about 7.2 mg enzyme.

3.7. Effects of water content

Water has a crucial role in enzymatic synthetic reactions in organic solvents, especially in the case of hydrolytic enzymes. All enzymatic reactions in organic solvents require some water, but the actual amount depends greatly on the enzyme. The lipases from different microbial sources need less than 1% water, whereas papain catalyses the esterification of *N*-protected amino acids with glycerol at a higher (10%) water content [13,14]. The dependence of the conversion on the water content described a maximum curve. The highest conversion was achieved at a water content of 2.87%.

3.8. Effects of pH

The pH of the reaction mixture has a considerable influence on the ionization state of enzymes and hence on the catalysis. The effect of pH on *N*-acetyl-L-arginine ethyl ester synthesis was studied in the pH range 3.0–9.3; 0.1 M sodium citrate/NaOH (pH 2–6) and 0.1 M triethanolamine/HCl (pH 6–9.3) buffers were used in the experiments. Fig. 2 shows the effects of pH on ester synthesis. The highest conversion of *N*-acetyl-L-arginine was mea-

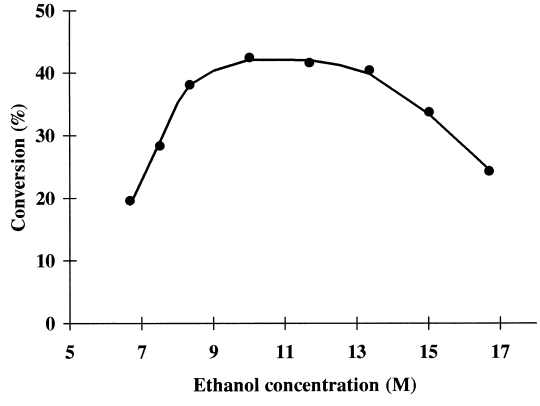

Fig. 1. Dependence of *N*-acetyl-L-arginine conversion on ethanol concentration. The reaction mixture contained 6.7–16.7 M ethanol, 7.2 mg trypsin, 5.0 mM *N*-acetyl-L-arginine, 125 μl 0.1 M triethanolamine/HCl buffer (pH 7.0) and 4 ml acetonitrile. The water content in the reaction mixture was 2.87%. The reactions were performed for 6 h at 30°C.

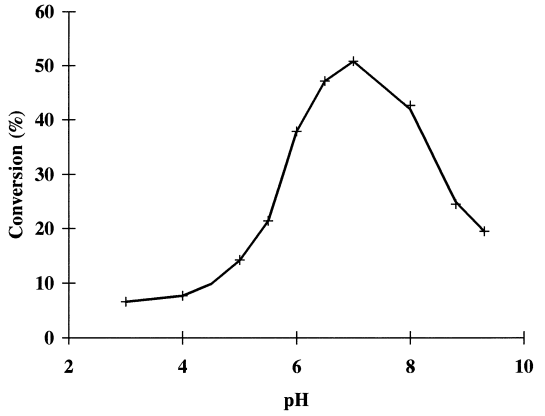

Fig. 2. Effects of pH on *N*-acetyl-L-arginine ethyl ester conversion. In the experiments, 125 μl 0.1 M sodium citrate/NaOH (pH 3.0–6.0) and 0.1 M triethanolamine/HCl (pH 6.0–9.3) buffers were used in the standard reaction mixture at a water content of 2.87%. The reactions were performed for 6 h at 30°C.

sured at pH 7. Not only the pH, but also the buffer itself influences the conversion. No ester formation was observed in 0.1 M phosphate buffer (pH 7.0), and lower conversions were found in 0.1 M Tris/HCl buffer at different pH values (data not shown). Yagisawa [15] studied the ester formation catalysed by bovine trypsin in 50% methanol for 1 h at 25°C between pH 4.4 and 4.8 and the yields were 24.6–35.5% for

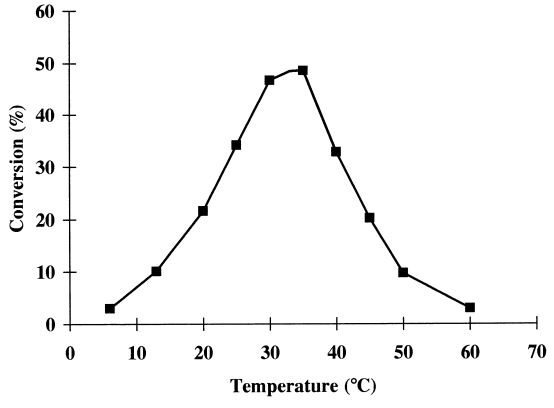

Fig. 3. Temperature dependence of *N*-acetyl-L-arginine ethyl ester synthesis. The reaction mixture contained 10.0 M ethanol, 7.2 mg trypsin, 5.0 mM *N*-acetyl-L-arginine, 125 μl 0.1 M triethanolamine/HCl buffer (pH 7.0) and 4 ml acetonitrile. The water content was 2.87%. The reactions were performed for 6 h at 6–60°C.

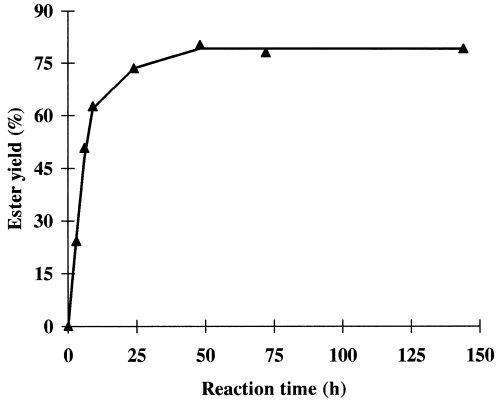

Fig. 4. Time dependence of *N*-acetyl-L-arginine ethyl ester yield under optimized conditions. For details, see text.

Bz-arginine substrate. The chymotrypsin-catalysed syntheses of *N*-acetyl-L-tyrosine esters in acetone furnished the highest yields at pH 7.0 [16]. Peptide synthesis with proteases was performed at various pH values, ranging from 3.2 to 7.0, depending on the enzymes and the substrates [17,18].

3.9. Effects of temperature

The effects of temperature on *N*-acetyl-L-arginine ethyl ester synthesis were studied in the temperature range 6–60°C. Maximal conversion was measured at 30–35°C (Fig. 3). Similar results were obtained by Shih et al. [5] in the esterification of *N*-protected amino acids with secondary alcohols, and by Ampon et al. [6] in a

Table 4
Changes in hydrolytic activity of trypsin during operation for 6 days
For details, see text.

Time (h)	Residual activity (%)
0	100.0
3	105.0
6	111.6
9	106.4
24	95.3
48	93.0
72	92.6
144	87.7

study of sugar esterification by alkylated trypsin in dimethylformamide. Transesterification and peptide synthesis reactions with trypsin were carried out mainly at 30–37°C [6,19].

3.10. Effects of reaction time

Under optimized reaction conditions, in acetonitrile with 5 mM *N*-acetyl-L-arginine, 10.0 M ethanol and 7.2 mg trypsin at 2.87% water content, pH 7.0 and 30°C, the synthesis of *N*-acetyl-L-arginine ethyl ester was followed for 6 days. At appropriate time intervals, samples were withdrawn from the reaction mixture and analysed for ester synthesis, and the residual hydrolytic enzyme activity at each time point was also measured. A conversion of 79.3% was achieved in the ethyl ester synthesis after 2 days and no further increase was obtained during longer incubation (Fig. 4). The changes in hydrolytic activity of trypsin are presented in Table 4. During the 6 days of operation, the activity of the enzyme decreased by only about 13%, which reveals that the acetonitrile did not inactivate the trypsin appreciably under these conditions. This phenomenon reveals an apparent equilibrium in the reaction medium. Similar results were found with carboxypeptidase A in dipeptide syntheses [20]. The esterification equilibrium of Bz-arginine catalysed by bovine trypsin corresponded to at 28% Bz-L-arginine methyl ester at pH 4.62 and 25°C in 50% methanol [15].

Our results indicate that bovine trypsin can be used for the direct esterification of *N*-acetyl-L-arginine with different alcohols in organic solvents; the highest yield is achieved with ethanol.

References

[1] C.R. Wescott, A.M. Klibanov, Biochim. Biophys. Acta 1206 (1994) 1.
[2] K. Martinek, A.N. Semenov, I.V. Berezin, Biochim. Biophys. Acta 658 (1981) 76.
[3] C.H. Wong, K.T. Wang, Experientia 47 (1991) 1123.
[4] V. Cerovsky, Biotechnol. Lett. 12 (1990) 899.
[5] I.L. Shih, L.C. Chiu, C.T. Lai, W.C. Liaw, D.F. Tai, Biotechnol. Lett. 19 (1997) 857.
[6] K. Ampon, A.B. Salleh, A. Teoh, W.M.Z. Wan Yunus, C.N.A. Razak, M. Bashri, Biotechnol. Lett. 13 (1991) 25.
[7] R.M. Blanco, J.M. Guisán, P.J. Halling, Biotechnol. Bioeng. 40 (1992) 1092.
[8] H. Hansen, Hoppe–Seyler/Thierfelder Handbuch der physiologisch-und pathologisch-chemischen Analyse, Springer, Berlin, 1966, p. 84.
[9] G.W. Schwert, Y. Takenaka, Biochim. Biophys. Acta 16 (1955) 570.
[10] K. Tanizawa, Y. Kanaoka, Chem. Pharm. Bull. 32 (1984) 4514.
[11] J.L. Vidaluc, M. Baboulene, V. Speziale, A. Lattes, P. Monsan, Tetrahedron 39 (1983) 269.
[12] M.-H. Remy, P. Bacou, C. Bourdillon, D. Thomas, Biochim. Biophys. Acta 911 (1987) 252.
[13] G. Fregapane, D.B. Sarney, E.N. Vulfson, Enzyme Microb. Technol. 13 (1991) 796.
[14] Y.V. Mitin, K. Braun, P. Kuhl, Biotechnol. Bioeng. 54 (1997) 287.
[15] S. Yagisawa, J. Biochem. 89 (1981) 491.
[16] K. László, L.M. Simon, Prog. Biotechnol. 15 (1998) 713.
[17] K.H. Lee, P.M. Lee, Y.S. Siaw, K. Morihara, Biotechnol. Lett. 14 (1992) 1159.
[18] S.I. Wayne, J.S. Fruton, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 3241.
[19] V. Schellenberger, U. Schellenberger, H.-D. Jakubke, N.P. Zapevalova, Y.V. Mitin, Biotechnol. Bioeng. 38 (1991) 319.
[20] A. Vértesi, L.M. Simon, J. Biotechnol. 66 (1998) 75.