Endopeptidases of *Bacillus subtilis* IBTC-3 and *B. alcalophilus* PB92 in synthesis of precursors of biologically active peptides

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Two endopeptidases (from *Bacillus subtilis* IBTC-3 and from *B. alcalophilus* PB92-commercial preparation) efficiently synthesized amino acid esters (NAc-Tyr-OEt and NAc-Phe-OEt) and dipeptides (NAc-Tyr-Gly-NH$_2$ and NAc-Tyr-Arg-NH$_2$) in organic solvent/water systems. The rate of NAc-Tyr-OEt synthesis mediated by the native subtilisin IBTC-3 was maximum (0.23 Umg$^{-1}$) in ethanol/5-7% w/v water system, while the highest activity of the freeze-dried enzyme (0.18 Umg$^{-1}$) was achieved, when water content was 9-10% w/v. The preferred system for dipeptide synthesis (using NAc-Tyr-OEt as acyl donor) by both the enzymes was acetonitrile/4% w/v water. In this system, the maximum yield of NAc-Tyr-GlyNH$_2$ was 71 and 80% and that of NAc-Tyr-Arg-NH$_2$ was 53 and 40% for subtilisin IBTC-3 and peptidase PB92, respectively. In contrast to the peptidase PB92, the subtilisin efficiently catalyzed esterification of NAc-Tyr with 1-butanol and isopropanol.

**Keywords:** Subtilisin, Alkaline peptidase PB92, Immobilization, Amino acid esters, Peptides, Non-aqueous enzymology

Numerous hydrolytic enzymes such as peptidases have been successfully employed as catalysts of reactions carried out in organic media with low water content. These reactions include esterification, inter- and transesterification, amidation, and peptide synthesis.$^{1-4}$ The application of organic solvents for hydrolyase-mediated reactions has the following advantages: (a) a shift of reaction equilibrium towards synthesis, (b) suppression of hydrolytic side-reaction, and (c) increased solubility of more hydrophobic reagents.$^5$

Studies on synthesis of amino esters and peptides have been carried out in hydrophilic monophasic systems, which provide better access of the enzyme to substrate, eliminate diffusion limitations at the interface and prevent gathering of product molecules in the environment of biocatalyst, which can lead to its inhibition, particularly when the volume of water phase is small. Moreover, the solubility of majority of amino acids, their derivatives and peptides in organic polar solvents is higher than in non-polar media.$^6$ Apart from monophasic reaction systems, biphasic media or reverse micelles have also found application in peptides and esters synthesis by proteolytic enzymes.$^7$ Some well known commercial endopeptidases like α-chymotrypsin, trypsin, Carlsberg and BPN’ subtilisins, papain, thermolysin (used for aspartame manufacturing)$^8$ and some others$^{9-12}$ have been frequently used for this purpose.

Kinetic and molecular properties of *Bacillus subtilis* IBTC-3 subtilisin, its biosynthesis in a semi-technical scale (with an average yield of 13500 Udm$^{-3}$) and simple purification procedure comprising affinity chromatography on Bacitracine-CNBr Sepharose and yielding the homogeneous biocatalyst have been described elsewhere.$^{13}$ We found that this enzyme efficiently catalyzes hydrolysis reaction of Tyr, Phe and Arg ethyl esters in aqueous medium. This paper reports on our further studies on two serine endopeptidases the subtilisin IBTC-3 and a commercial *B. alcalophilus* PB92 peptidase (Maxacal). Although a lot of work has been done to elucidate the peptidase PB92 structure, binding site details and consequences of its mutations as well as the mechanism of adaptation to alkaline environment,$^{14}$ its application for synthesis in organic media has not yet been reported.

This study has focused on the optimization of synthesis of amino esters (NAc-Tyr-OEt and NAc-
Phe-OEt) and dipeptides (NAc-Tyr-Gly-NH₂ and NAc-Tyr-Arg-NH₂) catalyzed by *B. subtilis* IBTC-3 subtilisin and *B. alcalophilus* PB92 endopeptidase, with respect to the solvent system, water concentration, form of biocatalysts (native, lyophilized or immobilized) and co-substrate concentration. Tyr-Gly is a fragment of an endogenous opioid pentapeptide Leu–enkephalin (Tyr-Gly-Gly-Phe-Leu) with morphine-like activity whereas Tyr-Arg, isolated for the first time from bovine brain is a precursor of endogenous neuropeptide kyotorphin which is involved in pain alleviation in brain.

**Materials and Methods**

**Reagents**

1,6-Diisocyanatohexane was obtained from Aldrich (USA). O-Diphostaldehyde, N-acetyl-L-phenylalanine (NAc-Phe), N-acetyl-L-tyrosine (NAc-Tyr), N-acetyl-L-phenylalanine ethyl ester (NAc-Phe-OEt), N-acetyl-L-tyrosine ethyl ester (NAc-Tyr-OEt), N-benzoyl-L-tyrosine ethyl ester (NBz-Tyr-OEt), glycine amide hydrochloride (GlyNH₂·HCl), arginine amide dihydrochloride (ArgNH₂-2HCl) and N-benzoyl-L-arginine ethyl ester (NBz-Arg-OEt), and trifluoroacetic acid (TFA) were purchased from Sigma (USA). Acetone and acetonitrile (from Baker, USA) were stored over molecular sieves 4 Å. Triethylamine was purchased from POCH (Poland) and bovine serum albumin from ICN Biomedicals (USA). Regenerated cellulose membranes were manufactured by Millipore (USA). Controlled-pore glass (CPG) 200/400 mesh, 480 Å was purchased from Serva (Germany). All other reagents were analytical grade.

**Enzymatic preparations**

Serine endopeptidases (EC 3.4.21.62): subtilisin IBTC-3 from *B. subtilis* IBTC-3 (the strain from the collection of Institute of Technical Biochemistry, Technical University of Lodz) and alkaline peptidase PB92 from *B. alcalophilus* (Maxacal, Gist Brocades) were used throughout the studies. Highly purified preparations of these enzymes were obtained as described elsewhere. Their esterolytic activities were (in Umg⁻¹): 1.0 and 11.8 (for NAc-Tyr-OEt), respectively at pH 8.0 and 30°C. The native preparations of subtilisin IBTC-3 and peptidase PB92 were obtained from their solutions in 0.1% (CH₃COO)₂Ca by using Amicon centrifuge filters, cut-off 10 kDa. The lyophilized powders of both the peptidases (IBTC-3Lyoph, PB92Lyoph) were obtained from the purified enzymes that were frozen at -40°C and lyophilized (-30°C, 0.37 mbar, 12 h).

**Immobilization of peptidase PB92**

A porous glass carrier was boiled in 5% HNO₃ for 45 min, washed and dried for 24 h at 115°C. 1 g of dry support was suspended in 5 cm³ of 1.6-diisocyanatohexane and incubated for 2 h at 30°C. After washing with acetone and distilled water, the activated carrier was added to the purified enzyme solution [1.2 mg cm⁻³ protein in 0.1% (CH₃COO)₂Ca] and gently shaken for up to 12 h at 6°C. It was subsequently filtered, washed with distilled water, 1 M NaCl, and 0.1% (CH₃COO)₂Ca, and dried at 10°C over P₂O₅. Approximately, 90% of protein was fixed to the carrier under these conditions.

**Synthesis of amino acid esters**

Standard reaction mixtures (2.5 cm³) contained NAc-Tyr or NAc-Phe (5.0 mM) dissolved in aliphatic alcohols (methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, pentan-1-ol). Water concentration in the monophasic reaction medium was 4-6% w/v. Esterification was initiated by addition of enzymatic preparation (native, lyophilized or immobilized). NAc-Tyr-OEt synthesis was also carried out in mixtures of ethanol and co-solvent (AC, ACN or hexane) mixed in the ratio 1:1 (v/v).

**Synthesis of dipeptides**

Concentrated solution of native enzyme (35 μg subtilisin IBTC-3 protein or 20 μg peptidase PB92 protein) was added to the mixture (2.5 cm³) of acyl donor NAc-Tyr (5 mM) or its ester derivative NAc-Tyr-OEt (5 mM) and GlyNH₂·HCl or ArgNH₂·2HCl (5-35 mM) (used as acyl acceptor), water (4% w/v), Et₃N (5-70 mM) and organic solvents such as: ethanol, AC, ACN, THF or ethanol-hexane mixture (1:1 v/v). Reactions were carried out in shaken closed vials (250 rpm) at 30°C. Aliquots (100 μl) were withdrawn periodically and filtered through PTFE membranes to remove the biocatalyst (to stop the reaction). The filtrates were evaporated and the solids were re-dissolved in a mobile phase and analyzed by HPLC as described below. The reference samples did not contain the biocatalyst. Concentration of reaction products was calculated on the basis of surface area below the peaks in chromatograms and standard curves. Reaction conditions are shown under respective Tables and Figures.

**Quantitative HPLC analysis**

HPLC analysis was performed using Knauer system equipped with a pump and UV/VIS detector. Sample
(50 µl) was injected on ODS-Hypersil column (250 × 4.6 mm) and eluted with water: ACN 70:30 (v/v) at a flow rate of 1 cm³·min⁻¹. Elution profiles were monitored for NAc-Tyr-OEt and NAc-Tyr at 280 nm, for NAc-Phe-OEt and NAc-Phe at 215 nm, and for NAc-Tyr-Gly-NH₂ and NAc-Tyr-Arg-NH₂ at 220 and 280 nm, respectively. For quantitative determination of products and/or the remaining substrates, the method with the internal standard was used. NAc-Tyr-Arg-NH₂ was assayed by using OPA reagent. The produced derivative was determined by HPLC using Shimadzu system equipped with SphEvi RP-18 column (220 × 2.1 mm) and fluorescent detector. The samples were eluted with a linear gradient (50-90%) of phase B (H₂O:ACN, 80:20, v/v) in phase A (25 mM CH₃COONa, pH 7.25) for 20 min at a flow rate of 0.3 cm³·min⁻¹. Elution profile was determined at 340 nm.

Enzymatic-synthesis of NAc-Tyr-Gly-NH₂ and NAc-Tyr-Arg-NH₂ standards
The reaction mixture (25 cm³) contained NAc-Tyr-OEt (50 mM) and GlyNH₂·HCl or ArgNH₂·2HCl (50-300 mM) and Et₂N (150-300 mM) (dissolved in ACN). Reaction of condensation was initiated by addition of the native peptidase PB92 (1 mg) in the case of NAc-Tyr-Gly-NH₂ synthesis or subtilisin IBTC-3 and alkaline peptidase PB92 of 27000 Da. One unit of synthetic activity (U) denoted 1 µmole of amino acid ester or dipeptide formed per min. The specific synthetic activity of peptidase was expressed in U per mg of protein. Protein concentration in the enzyme solutions was estimated according to Bradford using bovine serum albumin as standard. Water content in reaction mixtures was determined by Karl-Fischer method using a Mettler DL 18 moisture titrator.

³H NMR spectra of dipeptides

NAc-L-Tyr-Gly-NH₂
¹H NMR (DMSO-d₆, 250.13 MHz): 1.79 (3H, s, HCCONH); 2.62 (1H, dd, JAB=13.7, JAX=10.0, HOC₅H₄CH-CH); 2.87 (1H, dd, JAB=13.7, JBX=5.0, HOC₅H₄CH-CH); 3.51 (1H, dd, JAB=17.5, JAX=5.25, HNC₅CHCONH); 3.65 (1H, dd, JAB=17.5, JBX=5.0, HNC₅CHCONH); 4.30–4.35 (1H, m, HOC₅H₄CHCHNH); 6.62 (2H, d, JAB=8.2, HOC₅H₄CH₂); 7.01 (2H, d, JAB=8.2, HOC₅H₄CH₂); 7.09 (2H, d, J=15.0, CONH₂); 8.10 (1H, d, J=7.7, HCCONHCH); 8.18 (1H, t, J=5.25, HNC₅CHCONH); 9.18 (1H, s, HOC₅H₄CH₂). FABMS: 302 (M + Na)⁺.

NAc-L-Tyr-L-Arg-NH₂
¹H NMR (D₂O, 250.13 MHz): 1.49-1.59 i 1.77-1.80 (4 H, m, H₂CCH₂); 1.94 (3H, s, HCCCONH); 2.90-3.01 (2H, m, HOC₅H₄CHCH₂); 3.10-3.24 (2H, m, H₂CCH₂CH₂NH); 4.19-4.22 (H, m, HOC₅H₄CHCHNH); 4.46 (1H, t, HNC₅CHCONH); 6.84 (2H, d, JAB=7.5, HOC₅H₄CH₂); 7.15 (2H, d, JAB=7.5, HOC₅H₄CH₂). FABMS: 379 (M + H)⁺.

Estimation of optical purity and structure of synthesis products
Optical purity of dipeptide standards was determined by HPLC on enantioselective column EnantioPac. Elution was carried out at 25°C with water/acetonitrile (70:30, v/v) at a rate of 0.3 cm³·min⁻¹. Elution profiles of NAc-Tyr-Gly-NH₂ and NAc-Tyr-Arg-NH₂ were monitored at 220 and 280 nm, respectively.

Kinetic measurements
Kinetic constants Kₘ and Vₘₐₓ in the reaction of esterification were determined according to Hanes linearization method and kₘₐₓ was calculated for molecular weights of subtilisin IBTC-3 and alkaline peptidase PB92 of 27000 Da. One unit of synthetic activity (U) denoted 1 µmole of amino acid ester or dipeptide formed per min. The specific synthetic activity of peptidase was expressed in U per mg of protein. Protein concentration in the enzyme solutions was estimated according to Bradford using bovine serum albumin as standard. Water content in reaction mixtures was determined by Karl-Fischer method using a Mettler DL 18 moisture titrator.

¹H spectra (250 MHz) were recorded with a Bruker Avance DPX spectrometer using DMSO as solvent. Values of δ (chemical shift) were given in ppm, and that of J (coupling constant) in Hz. Mass spectra were obtained with fast atom bombardment as ionization method, FAB MS⁺ (Tg/Cu).

Separation of dipeptides standards
NAc-Tyr-Gly-NH₂ and NAc-Tyr-Arg-NH₂ (applied as standards) were synthesized as described above and purified by HPLC using LDC chromatograph equipped with Vydac ODS column (250 × 22 mm). Sample (500 µl) elution was carried out with a linear gradient 0-20% (v/v) of phase B (ACN/water/TFA, 90:10:0.038, v/v) in phase A (water/TFA, 100:0.05, v/v), at a flow rate of 16 cm³·min⁻¹. The product was detected at 220 nm.

Estimation of optical purity and structure of synthesis products
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(1H, s, HNCNHNH₂); 8.0 (1H, d, J=7.75, H₂CCONHCH); 8.1 (1H, d, J=8.5, HNCHCONH₂); 9.19 (1H, s, HOC₆H₄CH₂).

Results and Discussion

Synthesis of amino acid ethyl esters: Effect of water concentration and biocatalyst

Achievements of Klibanov and Dordick\textsuperscript{18-21} in the field of non-aqueous enzymology resulted in intensification of research into the enzyme-catalyzed processes in organic solvents. To display catalytic activity in organic solvents, the enzymes need small amounts of water, which participates in non-covalent interactions of the enzyme molecules and plays an important role in the dynamics of conformational changes, which are of particular importance during catalytic events\textsuperscript{22}. Therefore, the effect of water concentration in organic solvent systems on the yield of amino ester synthesis catalyzed by subtilisin IBTC-3 and endopeptidase PB92 was investigated in the present study. In our experiments, NAc-Tyr esterification was carried out in ethanol supplemented with 0.2-20% w/v water (Fig. 1).

The native subtilisin IBTC-3 was optimally active (0.23 Umg\textsuperscript{-1}) in the presence of 5-7% w/v water. However, the freeze-dried subtilisin IBTC-3 required the higher water concentration than the native enzyme and achieved the maximum activity (0.18 Umg\textsuperscript{-1}), when reaction mixture contained 9-10% w/v water. The freeze-dried preparations of chymotrypsin, subtilisin Carlsberg\textsuperscript{23,24} and trypsin\textsuperscript{25} display maximum activities when reaction mixtures contain 6-7 or 3% v/v water, respectively. The activity of the lyophilized subtilisin IBTC-3 was about 22% lower than that of the native preparation. Lyophilization brings about the reversible dehydration of proteins and can cause changes in their 3D structure which in turn may decrease the catalytic activity. Retrieval of the native enzyme conformation, which is essential for the catalytic activity, can be difficult in organic solvent even when water content is relatively high\textsuperscript{26}.

Bone's hypothesis\textsuperscript{22} suggests that protein-bound water molecules shield electrostatic interactions between polar and charged amino acid residues located in the active site, thereby increasing the flexibility of this fragment of the molecule and this in turn benefits the catalytic activity. When water concentration was higher than the optimum, the rate of ester synthesis decreased for both the preparations of subtilisin IBTC-3. At water concentration of 20% w/v, the lyophilized and native enzyme preparations retained 60 and 39% of maximum activity, respectively. Affleck and co-authors\textsuperscript{19} have shown that when water concentration is higher than that corresponding to the highest catalytic activity, the flexibility of enzyme molecules is increased leading to some subtle conformational changes, negatively affecting its catalytic activity. Furthermore, synthesis reactions catalyzed by hydrolases are usually negatively affected by excess of water due to hydrolysis of newly generated products. In case of peptidase-mediated reactions also, the enzyme autolysis is possible.

The dependence of enzyme activity in synthesis reactions (further referred to as the synthetic activity) on water concentration in reaction mixtures was also observed for the alkaline peptidase PB92 preparations, commercial or immobilized on porous-glass. Immobilized peptidase PB92 was the least sensitive of all the examined enzymatic preparations.

![Fig. 1—Effect of water concentration on the initial reaction velocity of NAc-Tyr-OEt synthesis catalyzed by soluble (ο-ο) or lyophilized (●-●) subtilisin IBTC-3 [Reaction conditions: Reaction mixtures contained NAc-Tyr (5.0 mM), ethanol mixed with water (2.5 cm\textsuperscript{3}) and 0.035 mg protein of subtilisin IBTC-3 (native or lyophilized). Values in parentheses show the activity of enzyme, expressed in Umg\textsuperscript{-1}].](image1)

![Fig. 2—Effect of water content on activity of immobilized (●-●) and commercial (ο-ο) preparations of peptidase PB92 [Reaction conditions: Reaction mixtures contained NAc-Tyr (5.0 mM), ethanol mixed with water (2.5 cm\textsuperscript{3}) and peptidase PB92 (0.1 and 0.4 mg of protein in the commercial and immobilized preparation, respectively)].](image2)
to fluctuations in water concentrations within the range between 1 and 20% w/v (Fig. 2). In nearly anhydrous ethanol (H₂O<0.01%), this enzyme displayed approximately 24% of maximum activity. Besides, the immobilized preparation of PB92 was characterized by high thermostability. Interestingly, the commercial peptidase PB92 was optimally active, when ethanol contained 6% w/v water and both a slight decrease or increase (±2% w/v) in this parameter brought about a significant (~60%) decrease in the synthetic activity. This drawback is thought to result from the presence of other components in the commercial preparation (it contains ~3 mg of protein per 1 g), whose function is to stabilize the enzyme when it acts in water²⁷. Probably, in organic medium, these agents can act as strong nucleophiles and provoke product (amino ester) hydrolysis.

**Effect of organic solvents on NAc-Tyr esterification**

Table 1 shows the effect of organic system (ethanol and co-solvents: hexane, AC and ACN) on catalytic features of the peptidases in reaction of NAc-Tyr esterification. Both the investigated enzymes displayed lower catalytic efficiency (two orders of magnitude) in synthesis reactions in comparison to hydrolysis in aqueous medium¹³. Catalytic efficiency of peptidase PB92 (kcat/Km of 350 M⁻¹s⁻¹) in ethanol, supplemented with 6% w/v water was higher than that of IBTC-3 subtilisin due to the relatively high affinity for NAc-Tyr (Km of 0.75 mM) and catalytic constant of its conversion to ester (kcat of 0.26 s⁻¹). Addition of co-solvents to ethanol decreased the kcat value for both the biocatalysts and the substrate affinity of peptidase PB92. However, under these conditions, subtilisin IBTC-3 displayed approximately 4-fold higher affinity for NAc-Tyr and slightly increased efficiency in NAc-Tyr-OEt synthesis (with an exception of acetone). Besides, this enzyme displayed 10-fold greater affinity for NAc-Phe as the substrate for esterification in ethanol than peptidase PB92 (Table 1, sample 5).

Subtilisin IBTC-3 effectively catalyzed esterification of NAc-Tyr with primary aliphatic alcohols containing 2-4 carbon atoms and with the secondary alcohol 2-propanol (Table 2). In case of peptidase PB92, the yield of NAc-Tyr conversion to corresponding esters decreased significantly with an increase in the length of alcohol chain. Both the enzymes were incapable of efficient NAc-Tyr-OMe synthesis, when reaction medium contained high concentrations of methanol. Methanol inactivated some other serine peptidases and horseradish peroxidase¹⁸,²⁸.

**Synthesis of dipeptide derivatives – the choice of acyl donor and acceptor**

The main advantages of the enzymatic peptide synthesis include the high enantioselectivity of the enzymes, lack of necessity of side-chain protection and the use of mild reaction conditions. Application of endopeptidases for peptide bond synthesis requires selection of the proper acyl donor (which fits to the endopeptidase’s S1 subsite), the nucleophilic acyl acceptor (which can be accommodated in the S1’ cavity of the enzyme) and the solvent. Generally, the organic medium should provide both good solubility of substrates and high stability of biocatalysts. Our earlier studies have revealed that NAc-Tyr is accepted as a substrate in the specificity pocket S1 of both the peptidase PB92 and subtilisin IBTC-3¹³.

Formation of peptide bonds mediated by chemical or biological catalysts requires activation of carboxylic group in acyl donor. Amino acid ester

### Table 1—Kinetic constants of peptidases B. subtilis IBTC-3 and B. alcalophilus PB92 in amino acid esters synthesis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent/co-solvent</th>
<th>kcat (mM)</th>
<th>kcat /Km (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IBTC-3</td>
<td>PB92</td>
<td>IBTC-3 PB92</td>
</tr>
<tr>
<td>1</td>
<td>Ethanol</td>
<td>4.00</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>Ethanol/AC</td>
<td>1.22</td>
<td>4.70</td>
</tr>
<tr>
<td>3</td>
<td>Ethanol/ACN</td>
<td>0.87</td>
<td>1.34</td>
</tr>
<tr>
<td>4</td>
<td>Ethanol/Hexane</td>
<td>1.10</td>
<td>2.29</td>
</tr>
<tr>
<td>5</td>
<td>Ethanol</td>
<td>0.85</td>
<td>8.42</td>
</tr>
</tbody>
</table>

*Reaction conditions:* (Samples 1-4) NAc-Tyr (2.5-20 mM) or (sample 5) NAc-Phe (2.5-20mM), subtilisin IBTC-3 (0.035mg) and peptidase PB92 (0.020mg), EtOH co-solvent (1:1 v/v) and H₂O (6 % w/v), total volume of 2.5 cm³, 30°C.

### Table 2—Yield of synthesis of NAc-Tyr esters

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilisin IBTC-3</td>
<td>Peptidase PB92</td>
</tr>
<tr>
<td>Methanol</td>
<td>2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>92</td>
</tr>
<tr>
<td>Propan-1-ol</td>
<td>70</td>
</tr>
<tr>
<td>Propan-2-ol</td>
<td>62</td>
</tr>
<tr>
<td>Butan-1-ol</td>
<td>63</td>
</tr>
<tr>
<td>Butan-2-ol</td>
<td>Nil</td>
</tr>
<tr>
<td>Pentanol-1-ol</td>
<td>Nil</td>
</tr>
</tbody>
</table>

*Reaction conditions:* NAc-Tyr (5 mM), alcohol mixed with 4-6% w/v water (2.5 cm³), concentrated solution of subtilisin IBTC-3 or peptidase PB 92 (0.1 mg protein), 30°C, 24 h.
derivatives are commonly used as activated acylating agents in peptide synthesis. The relatively high esterolytic activity of the majority of endopeptidases speeds up the first step of peptides synthesis—acylation. It is to note that only in the case of these endopeptidases, which catalyze peptide synthesis according to the kinetically controlled mechanism, the ratio of rate constants of transfer and hydrolysis reactions ranges between $10^2$ and $10^4$ values$^{29,30}$. Kinetic control of reactions, which applies to serine and cysteine endopeptidases suggests that the reaction is initiated by donor hydrolysis, followed by the rapid formation of covalent acyl-enzyme intermediate. This intermediate donates the acyl group either to the amino acceptor or water. Under kinetic control conditions, the target peptide product is accumulated as long as the acyl donor ester is available.

The yield of NAc-Tyr-Gly-NH$_2$ formation in ethanol, catalyzed by peptidase PB92 was 67% when NAc-Tyr-OEt was used as the acyl donor and only 14% when the latter was replaced by NAc-Tyr. This difference provided evidence that the kinetically controlled peptide bond synthesis is usually more effective than the thermodynamically controlled one. In the thermodynamically controlled reaction, the reaction equilibrium had to be displaced in favor of synthesis by using organic co-solvents or through product precipitation or extraction.

**Effect of water and Gly-NH$_2$ concentrations on NAc-Tyr-Gly-NH$_2$ synthesis**

Competition between two nucleophiles, i.e. amino acid and water for acyl-enzyme intermediate produced through the deacylation step led to formation of dipeptide (P) and the product of acyl-enzyme hydrolysis (H). When NAc-Tyr-Gly-NH$_2$ (P) synthesis was conducted in ethanol, the rate of aminolysis increased with increase in water concentration from 0.2 to 4% (w/v), but the further increase in the latter resulted in excessive NAc-TyrOH (H) formation (Table 3). Also, it is reported that aminolysis rate of α-chymotrypsin catalyzed Bz-Tyr-Val-NH$_2$ synthesis in ACN increases abruptly in medium containing 4-5% (v/v) water$^{31}$. At higher water concentration, there was no increase in the activity of this enzyme, because water acted only as a nucleophile in acyl-donor hydrolysis. Earlier report suggests that water concentration of approximately 5% is optimal for systems containing polar organic solvents$^{32}$.

The P/H ratio is considered as the best determinant of peptide synthesis efficiency. To enhance the aminolysis and minimize the hydrolysis, it is necessary to adjust also the concentration of amino nucleophile. In our case, hydrochlorides of GlyNH$_2$ and ArgNH$_2$ were used as acyl acceptors. Their nucleophilicity and solubility were increased by supplementing with Et$_3$N (the deprotonating agent). An excess of acyl acceptor suppressed the side reaction of hydrolysis during the deacylation step and increased the yield of peptide synthesis. Besides, the higher molar ratio of acceptor to donor prevented inhibition of the enzyme by the substrate.

The enzymatic aminolysis reactions were also conducted in ethanol-4% w/v water system at molar ratio of GlyNH$_2$: NAc-Tyr-OEt ranging between 1 and 7 (Fig. 3). For equimolar concentrations of both the

<table>
<thead>
<tr>
<th>Water (% w/v)</th>
<th>Specific activity (Umg$^{-1}$)</th>
<th>Yield (%)</th>
<th>P/H</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAcTyrGlyNH$_2$ (P)</td>
<td>NAcTyrOH (H)</td>
<td>NAcTyrGlyNH$_2$ (P)</td>
<td>NAcTyrOH (H)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.17</td>
<td>0.022</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>0.047</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>0.28</td>
<td>0.100</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>0.23</td>
<td>0.140</td>
<td>27</td>
</tr>
</tbody>
</table>

**Reaction conditions**: NAc-Tyr-OEt (5 mM), GlyNH$_2$·HCl (15 mM), Et$_3$N (15 mM), native enzyme preparation (35 µg protein), (E:S molar ratio of 1: 10000), EtOH (2.5 cm$^3$), 30°C, 24 h.
substrates, the enzymatic activity and P/H ratio were 0.13 Umg\(^{-1}\) and 2.8, respectively. The seven-fold excess of GlyNH\(_2\) versus NAc-Tyr-OEt resulted in 3.5-fold greater catalytic activity, while the synthesis yield (Yield\(_{24}\)) increased from 22 to 48% (Fig. 3).

**Influence of various organic solvents on NAc-Tyr-Gly-NH\(_2\) synthesis**

NAc-Tyr-Gly-NH\(_2\) and NAc-Tyr-Arg-NH\(_2\) synthesis was conducted in hydrophilic solvents such as ACN, AC, THF and the mixture of ethanol and hexane (Tables 4 and 5). Irrespective of the solvent, the two dipeptides were the major products in reaction mixtures and by-products derived by undesired oligomerization were not detected by chromatography methods.

Among the solvents, ACN (an aprotic solvent) was the most suitable for NAc-Tyr-Gly-NH\(_2\) synthesis, because it gave the highest product yield (71 and 80%) and P/H ratio (7.1 and 10.0) for subtilisin IBTC-3 and peptidase PB92, respectively (Table 4). ACN has been frequently used as reaction medium for enzymatic synthesis of polar compounds. For example, in ACN system, the yield of NAc-Tyr-Gly-NH\(_2\) synthesis catalyzed by subtilisins Carlsberg and BPN’ was found to be 78 and 60%, respectively. In our experiments, the similar yield was achieved by using approximately 200-fold lesser amount of the enzyme preparation. Generally, aprotic solvents are known to enhance the nucleophilic potential of acyl acceptor. In these solvents, the charged nucleophile is less solvated, while in protic solvents, it forms hydrogen bonds, making nucleophilic attack more difficult.

Relatively high peptide synthesis yield was also achieved in ethanol:hexane (1:1). But, despite the high P/H ratio, the acyl-enzyme hydrolysis was also appreciable in this medium and this significantly decreased the effectiveness of condensation of substrates. It possibly resulted from the presence of larger amount of accessible water molecules, weakly interacting with hydrophobic hexane. In AC and THF, both the peptidases displayed very weak catalytic activity.

The yield of kyotorphin derivative (NAc-Tyr-Arg-NH\(_2\)) synthesis (53% and 40% for subtilisin IBTC-3 and peptidase PB92, respectively) was also the highest in ACN (Table 5). The higher yield of NAc-Tyr-Arg-NH\(_2\) (in comparison to our results) was achieved by Sareen in the system containing several-fold higher enzyme activity and significantly greater amount of ArgNH\(_2\) versus NAc-Tyr-OEt. When this reaction was catalyzed by glycosylated subtilisin immobilized on celite, the highest product yield was achieved in hexane-EtOH (40:60 containing 6% of water).

**Conclusion**

The study demonstrated two serine endopeptidases subtilisin IBTC-3 and alkaline peptidase PB92 as promising biocatalysts for synthesis of amino acid esters and dipeptides (precursors of biologically active peptides).
peptidases (E.C. 3.4.11.2) in organic solvents. Subtilisin IBTC-3 was found to be the more efficient biocatalyst in synthesis of kyotorphin derivative (N-acetyl-Tyr-Arg-NH$_2$) and esters of tyrosine and mid-chain alcohols. The activity in synthesis of peptide bonds by these two peptide hydrolases depended on water content in reaction medium. The enzymes acting in organic media are frequently characterized by a bell-shaped profile of the plot representing their synthetic activity as a function of water concentration. Our studies revealed that the shape of this plot and the optimum concentration of water in the organic solvent were different for various preparations of the biocatalysts. ACN was found to be the most suitable solvent for dipeptides synthesis by both subtilisin IBTC-3 and alkaline peptidase PB92.

Acknowledgments

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References