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Proteinases as catalysts in peptide synthesis

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ABSTRACT Proteolytic enzymes application as efficient catalysts of peptide synthesis is discussed. Serine proteinases - subtilisin and chymotrypsin turned to be especially efficient for kinetically controlled synthesis by condensation of peptide esters and peptide derivatives with free amino groups. Pepsin suits well to catalyze peptide bond formation according to equilibrium controlled procedure. Backward reaction - peptide bond hydrolysis might be efficiently suppressed if the synthesis is performed in organic solvents with low water content using as a catalyst a proteolytic enzyme sorbed on silica surface. Enzymatic synthesis of a series of hemostasis enzymes substrates and of laminin pentapeptide fragment is described.

Proteinases as any other catalysts are capable of accelerating both direct and reverse reactions, i.e., the hydrolysis as well as the synthesis of peptide bonds. The enzymatic synthesis became a practically significant way to prepare middle-sized peptides. Recently the attempts have been made to extend the method to the preparation of longer peptides and even small proteins (1).

Two main approaches are used to exploit this capacity of proteinases. According to the first one that corresponds strictly to reversal of the proteolysis the success of the synthesis depends on the equilibrium position, the latter being governed by the product precipitation, its transfer to another phase or exclusion of water from the reaction medium. The application of proteinases distributed over a support surface opens new prospects for this method, allowing drastic reduction of water content in the reaction mixture. The second approach requires preactivation of one of the reaction components, usually, the conversion of a carboxyl moiety into a more or less active ester. This helps to overcome thermodynamic problems however it requires stoppage of the reaction before the backward process - hydrolysis of the newly formed peptide bond - becomes noticeable. This "kinetically controlled" synthesis, when performed by a sorbed enzyme in organic solvent, at high conversion rate becomes transformed into an equilibrium controlled process.

The choice of an enzyme suitable for the synthesis depends on several factors. Its specificity is of importance, although, being not too stringent, it rarely represents a crucial factor, so far as rather large relative amount of the enzyme is usually introduced into the reaction and the duration of the latter is as a rule long enough. Both these factors allow to overcome the kinetical problems. Therefore, the enzyme can be used to catalyze the formation of peptide bonds that can hardly be considered as favorable ones for its specificity requirements. Thus, Phe-Arg, Phe-homophenylalanine, Phe-o-nitrotyrosine, Phe-S-methylcysteine peptide bonds were synthesized by pepsin as shown by C.A.A.Abdel Malak, whereas Ser-Arg and Arg-Arg bonds were formed by subtilisin as found by Terent'eva, Novgorodova *et al* in this laboratory. Hence, proteolytic enzyme binding sites are tolerant enough and the enzyme specificity, although it cannot and should not be neglected, only rarely presents an insurmountable obstacle, providing that the experimenter disposes a sufficiently broad palette of the enzymes. The following table based on the results

Table. Peptide bonds and the enzymes that catalyze their synthesis

Enzyme	Amino acid residues at positions	
	P_1	P_1'
Thermolysin	Ala, Leu, Phe, Thr, Asp, Pro, Gly	Phe, Leu, Ile Val, Met
Subtilisin 72	Ala, Leu, Phe, Trp, Tyr, His, Met, Ser, Arg, Val, Ile	Phe, Leu, Ala, Arg, Lys, Nle, Val, Ile, Met, Nva, Pro, D-Phe, NH_2-CH_2-
Pepsin	Phe, Leu, Trp, Met, Glu, Tyr	Phe, Leu, Arg, Ala, Val, Nle, Trp, Tyr
Chymosin	Phe, Leu	Phe, Leu, Ala, Nle, Met

gained in our laboratories illustrates an amazingly broad application range of the enzymatic peptide synthesis and indicates that proteolytic enzymes can carry out peptide bond formation also between such amino acid residues, which could be hardly considered as preferred ones.

One important factor has to be taken in consideration, although. Proteolytical enzymes as a rule possess extended substrate binding sites that are capable of accommodating 6-8 amino acid residues. Therefore, their application to catalyze peptide bond formation between e.g, two amino acids, being interesting *per se*, often gives the results hardly relevant for genuine evaluation of the method capacity.

It has been mentioned that, as a rule, large quantities of a proteinase are applied for peptide synthesis, enzyme:substrate molar ratio often attaining 1:10000 and even lower values that not only might cause economical problems, especially at scaling up, but, what is even more important, can induce unwanted side reactions. This phenomenon is explained by several reasons, including:

- diminishing of the enzyme activity due to the presence of relatively high concentrations of organic solvents added to enhance the reactants solubility;
- an enzyme inhibition with the peptides present in the mixture, especially with the product;
- an enzyme entrapment by the precipitated product - a phenomenon observed in this laboratory in the course of pepsin-catalyzed synthesis of middle-sized peptides (2);
- misoriented sorption of an enzyme on the support or formation of the enzyme clumps, where only exposed molecules can serve as catalysts etc.

Enzyme stability and specificity in the course of the enzymatic peptide synthesis deserve special attention. Generally, proteolytic enzymes applied to this end reveal amazingly high stability towards rather harsh conditions used for the synthesis, including high concentration of organic solvents. It should be stressed, however, that these enzymes as a rule belong to rather simple proteinases not endowed with quaternary structure, sophisticated domainial organization etc. The behavior of these proteinases as the catalysts even in organic solvents with very low water content implies that their essential structural and functional traits remain almost unchanged. It should be recalled that in organic solvents subtilisin (3) and chymotrypsin (4), as shown by X-ray analysis, retain the main traits of their spatial structure found in water, whereas a majority of hydration water molecules occupy the same positions as in water-saturated crystals. Although a few organic solvent molecules were bound by the enzyme that could influence its specificity, e.g., preferences toward hydrophobic moieties of the substrate, one could hardly await any radical changes in the enzyme specificity. These data refer to the enzyme crystals. It seems that proteinases

distributed over the surface of a support, e.g., of macroporous silica, such as silochrom or macroporous glass, are even more stable due to retention of the water molecules bound to the support surface. In our laboratory it was shown that subtilisin and alfa-chymotrypsin are indeed sorbed by macroporous silica support, whereas, pepsin does not shown proper signes of an adsorption by these supports, being easily eluted with water (5). Nevertheless, distribution of pepsin over the support surface helped to increase its efficiency in the course of enzymatic peptide synthesis. Practically relevant stability of sorbed subtilisin has been demonstrated in our laboratory by its repeated (up to 10 times) application as a catalyst (6). Of course, proteolytic enzymes stability in concentrated organic solvents should not be overestimated so far as among a number of the enzyme molecules introduced in the reaction mixture a good deal might turn inactive. We presume that there exist two states of the enzyme - the active (and similar in many aspects to that observed in water solutions) and the inactive (perhaps, retaining the capacity to be reactivated) rather than an array of different states each endowed with a different functional, especially specificity, characteristics.

A large series of peptides and their derivatives has been prepared in our laboratories, using pepsin, chymosin, subtilisin, trypsin and metalloproteinases as the catalysts.

ASPARTYL PROTEINASES

Pepsin was applied for equilibrium controlled peptide synthesis. This proteinase turned especially efficient in the synthesis of middle-sized peptides (2,7,8). Certain amino acid derivatives, like esters or, better, p-nitroanilides can be used as amino components to form peptide bond with N-protected peptides, e.g.

$$\text{Z-Ala-Ala-Phe-OH} + \text{H-Arg-NHC}_6\text{H}_4\text{NO}_2 \xrightarrow{\text{pepsin}} \text{Z-Ala-Ala-Arg-NHC}_6\text{H}_4\text{NO}_2$$

Several longer peptides have been successfully obtained using pepsin as a catalyst. It was observed, however, that these syntheses might be complicated by the enzyme entrapment into the gel of the peptide formed in the course of the synthesis. Further collapse of the gel leads to the enzyme inclusion into the precipitate, which strongly reduced its activity in the supernatant, although the entrapped enzyme is not inactivated (2). This effect enforces application of increased amount of the enzyme to compensate for the activity losses and makes necessary separation of the product from the admixture of pepsin. Apparently this phenomenon depends on the length of the peptide product as well as on the rate of its formation. To extend the application range of the method to longer peptides it turned useful to perform the synthesis in concentrated urea solutions containing also DMF necessary to keep the reactants in the solution (7). Remarkably, pepsin preserves its activity even in 4-8 M urea as well as in concentrated guanidinium chloride or in the presence of sodium dodecylsulphate. It is tempting to suppose that proteolytic enzymes designed to cleave the loose parts of protein substrates are substantially more stable toward denaturing conditions than the majority of other proteins. Chymosin - an enzyme homologous to pepsin turned to be useful as a catalyst of peptide bonds formation (9). In our opinion, pepsin and related aspartic proteinases deserve more attention as promising tools for peptide synthesis, including their application in organic solvents.

METALLOPROTEINASES

Thermolysin as well as another metalloproteinase isolated in our laboratory from *Bacillus megaterium* culture filtrate turned to be an efficient catalyst of peptide

bond formation under conditions of equilibrium-controlled synthesis. E.g., a number of chromogenic substrates for proteinases have been prepared by the reactions similar to the following one:

$$\text{Z-Ala-Ala-OH} + \text{H-Leu-NHC}_6\text{H}_4\text{NO}_2 \xrightarrow{\text{Thermolysin}} \text{Z-Ala-Ala-Leu-NHC}_6\text{H}_4\text{NO}_2$$

Again, one can introduce an organic solvent to improve the reactants solubility and, when necessary, to apply a metalloproteinase distributed over the support surface while performing the synthesis in essentially water-free organic solvent.

SUBTILISIN AND OTHER SERINE PROTEINASES

Serine proteinases are especially suitable for kinetically controlled peptide synthesis so far as the rate of acyl-enzyme formation and its further interaction with an amino component by far exceed the rate of the reverse reaction - peptide bond hydrolysis.

Subtilisin 72 that is closely related to the well characterized subtilisin Carlsberg has been purified in our laboratories by affinity chromatography and extensively used as a catalyst. It is rather efficient catalyst of peptide bond formation in water-organic solvent mixtures, especially in the cases when the product precipitates from the reaction mixture, thus reducing the danger of backward hydrolysis. More successful is the application of subtilisin distributed over the surface of macroporous silica, e.g., silochrom, the reaction being performed in organic solvents with very low water content. Under these conditions extremely low water activity minimizes the probability of backward hydrolysis reaction, whereas preactivation of the acylating component provided for efficient acceleration of the synthesis reaction. Hence, this methods combines the positive traits of both kinetically and equilibrium controlled procedures.

This procedure was successfully applied to synthesis of a series of peptides terminated with arginine or lysine p-nitroanilides or amides (10, 11). The synthesis of the latter compounds was complicated by repetitive attachment of arginine residues to the peptide C-terminus, apparently due to accumulation of hydrophilic arginine amide in the vicinity of the enzyme (M.P.Yusupova, S.A.Novgorodova).

Laminin pentapeptide fragment YIGSR and its derivatives were also prepared (6):

$$\text{Boc-Ile-Gly-Ser-OCH}_3 + \text{H-Arg-NHC}_6\text{H}_4\text{NO}_2$$
$$\downarrow \text{Subtilisin}$$
$$\text{Boc-Ile-Gly-Ser-Arg-NHC}_6\text{H}_4\text{NO}_2$$
$$\downarrow \text{CF}_3\text{COOH}$$
$$\text{Ile-Gly-Ser-Arg-NHC}_6\text{H}_4\text{NO}_2$$
$$\downarrow + \text{Ac-Tyr-OC}_2\text{H}_5\text{, chymotrypsin}$$
$$\text{Ac-Tyr-Ile-Gly-Ser-Arg-NHC}_6\text{H}_4\text{NO}_2$$
$$\downarrow \text{Trypsin}$$
$$\text{Ac-Tyr-Ile-Gly-Ser-Arg-OH}$$

A remarkable feature of serine proteinases specificity that makes these enzymes especially attractive as peptide synthesis tools is their capacity to cleave and, hence, to form not only peptide bonds between two amino acid residues, but to operate on a variety of peptide bond analogs. This capacity was utilized to prepare new peptide derivatives, like peptidyl amino alcohols and peptidoaldehydes. To this end N-protected peptide esters were reacted with the excess of amino alcohols with fairly good yields. Subsequent mild oxidation, e.g., by DMSO and acetanhydride leads to peptidoaldehydes useful as inhibitors and important synthones.

$$\text{Z-Ala-Ala-LeuOCH}_3 + \text{H-Phe-ol} \longrightarrow \text{Z-Ala-Ala-Leu-Phe-ol} \longrightarrow \text{Z-Ala-Ala-Leu-Phe-al}$$

(Phe-ol = $NHCH(CH_2C_6H_5)CH_2OH$; H-Phe-al = $NHCH(CH_2C_6H_5)CH{=}O$)

It turned possible as well, to acylate with peptide esters protected amino aldehyde derivatives thus obtaining peptidoaldehydes using even a less complicated route.

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