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USE OF METHYL RED IN SOLID PHASE SYNTHESIS

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We tested the methyl red dye as a chromogenic marker of various structures synthesized on a solid phase. A methyl red labeled substrate of pepsin (aspartic protease), which was immobilized on Perloza™ cellulose beads, was used for the quantification of presence of pepsin in biological materials. Methyl red was used also in another field of solid phase synthesis: an one bead - one structure library approach. It was used as a chromogenic marker for a digital coding of randomly generated molecular structures. This indicator proved to be useful for the quantification in biological tests as well as for an identification of structures from a library approach.

INTRODUCTION

We used methyl red in two completely different fields of solid phase applications. In one case the azo-dye was utilized as a chromogenic marker for labeling of N-terminus of substrates bound to Perloza™ beads (1). This arrangement seems to be more efficient for routine quantification of aspartic proteases in various biological materials (liquor, urine, milk etc.), than the commonly used hemoglobin assay (2) or the Phe(p-NO_2) method (3). An enzymatic cleavage (released dye) in our method can be followed spectrometrically in the visible range.

One bead-one structure library approach (4) utilizes various types of coding which enable structure elucidation of randomly generated nonpeptidic and/or nonsequenceable structures. Differently colored derivatives can provide variety of miscellaneous coding units with different properties. We used the methyl red dye as a model chromogenic label for so-called digital coding (4,5).

RESULTS AND DISCUSSIONS

Synthesis and evaluation of the substrate specific for pepsin

The Perloza™ (beaded cellulose) was modified by oxidation with periodate, followed by reductive amination with hexamethylene diamine in the presence of natrium borohydride. The amino group introduced was used for classical amide bond formation. As the first amino acid Fmoc protected Gly was coupled (nonacylated amino groups were acetylated) resulting in 0.44 mmol of amino groups per gram of dried Perloza™. The substrate sequence (KPAEFFAL) was synthesized by usual SPPS Fmoc/Bu^t strategy and DIPCDI/HOBt coupling procedure. Finally, the methyl red was coupled to N-terminus of the prepared substrate by the DIPCDI/HOBt activation. Side chain deprotection was accomplished under mild conditions (50% TFA/DCM, 10 min) due to very low stability of beaded cellulose in TFA. The synthesis was checked by the Kaiser test and purity of the substrate was proven by amino acid analysis and Edman sequencing degradation (before endcapping with methyl red).

The slurry of beads in a formate buffer (pH 2.3) was divided by pipetting of equimolar pools (defined by quantitative amino acid analysis per volume of slurry). We used pipetting of the slurry, because the microporous structure of the Perloza™ beads is lost on drying. Incubation of the bound labeled substrate was carried out with pig pepsin at 37°C for 16h in 1ml of total volume. Our observations showed that the substrate linked on cellulose beads is well accessible for the enzymatic cleavage and the relationship between released dye and amount of the immobilized substrate follows a curve which is typical for enzymatic reactions (Michaelis-Menten kinetics). The sensitivity of our assay is comparable to the hemoglobin assay (in the range of sub-micrograms to tenth of micrograms of pepsin in 1 ml of tested solution) but in contrast to this assay, our assay is simpler and released dye is measurable in a visible range (at 485 nm). We examined this quantification approach on the pepsin as a typical representative of aspartic proteases.

Utilization of methyl red in a model of digital coding of structures in a combinatorial libraries

Various combinatorial synthetic approaches have required many tricks for determination of randomly generated structures. We tested the use of methyl red as a chromogenic marker for tags with different physico-chemical properties (hydrophobicity, hydrophilicity) which can code molecular structures. As a model we prepared methyl red labeled -Lys(Ac)-NH_2 and -Lys-Lys-NH_2. Both molecules released from the single bead were clearly detectable by naked eye on TLC plate ($CHCl_3$/MeOH/pyridine) as red points (sensitivity about 50 pmol). Preparation of a random pool of molecules bound to beads includes orthogonal linkage enabling a cleavage of a coding molecule after identification of positively tested beads bearing an active compound. The coding molecule is assembled from a chromogenic part of a molecule (detectable in visible range, UV range, by fluorescence etc.) and a part responsible for various physico-chemical properties which make the separation of different tags possible. Acylation of the molecule by almost unlimited variety of carboxylic acids makes the fine tuning of the retention characteristics of the coding molecule very simple. TLC identification of the tag molecules allows for parallel evaluation of large number of codes, as well as for checking and identification of the tag by two independent methods – coding molecule can be evaluated by mass spectroscopic analysis of a molecule retrieved from the TLC plate.

CONCLUSIONS

We proposed utilization of a chromogenic label in two different approaches based on SPS both in quantification of an enzyme in biological materials and in a combinatorial approach.

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