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Selective chromogenic and fluorogenic peptide substrates for the assay of cysteine peptidases in complex mixtures

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ABSTRACT

This study describes the design, synthesis, and use of selective peptide substrates for cysteine peptidases of the C1 papain family, important in many biological processes. The structure of the newly synthesized substrates is Glp-Xaa-Ala-Y (where Glp = pyroglutamyl; Xaa = Phe or Val; and Y = pNA [*p*-nitroanilide], AMC [4-amino-7-methylcoumaride], or AFC [4-amino-7-trifluoromethyl-coumaride]). Substrates were synthesized enzymatically to guarantee selectivity of the reaction and optical purity of the target compounds, simplifying the scheme of synthesis and isolation of products. The hydrolysis of the synthesized substrates was evaluated by C1 cysteine peptidases from different organisms and with different functions, including plant enzymes papain, bromelain, ficin, and mammalian lysosomal cathepsins B and L. The new substrates were selective for C1 cysteine peptidases and were not hydrolyzed by serine, aspartic, or metallo peptidases. We demonstrated an application of the selectivity of the synthesized substrates during the chromatographic separation of a multicomponent set of digestive peptidases from a beetle, *Tenebrio molitor*. Used in combination with the cysteine peptidase inhibitor E-64, these substrates were able to differentiate cysteine peptidases from peptidases of other classes in midgut extracts from *T. molitor* larvae and larvae of the genus *Tribolium;* thus, they are useful in the analysis of complex mixtures containing peptidases from different classes.

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Peptide hydrolases of the C1 (papain) family belong to clan CA of cysteine peptidases containing a catalytic diad, Cys and His. Two other active site residues are found: a Gln residue preceding the catalytic Cys and an Asn residue following the catalytic His. The Gln residue is believed to help in the formation of the "oxyanion hole," and the Asn is believed to orientate the imidazolium ring of the catalytic His [1–3]. Cysteine peptidases have been identified and studied in detail in different organisms—viruses [4,5], bacteria [6,7], protozoa [8–16], plants [1,17–21], and mammals [3,17,22,23]. The ancestor of this family, a plant peptidase papain from papaya, as well as closely related peptidases from the tropical fruits bromelain (pineapple) and ficin (fig latex) have important applications in the food industry, pharmacology, and scientific research [1,24,25]. A large group of C1 family cysteine peptidases are lysosomal cysteine cathepsins, described mainly in mammals and humans [3,26,27]. Cysteine cathepsins are responsible for nonselective lysosomal protein degradation, but they also participate in more specific processes such as activation of zymogens, hormone maturation, and antigen presentation, among others. However, cysteine peptidases also are indicated in the development of different pathologies such as osteoporosis, rheumatoid arthritis, atherosclerosis, cancer metastasis, and tumor invasion [26,28–35]. Digestive cysteine peptidases also have been described in some insect pests, including some families of beetles, aphids, and thrips [36–45].

Given the fact that cysteine peptidases are widespread and important, highly specific and effective peptide substrates are needed. Although cysteine peptidases of the papain family have broad substrate specificity, activity assays for these enzymes have been limited to a set of Arg-containing substrates, starting from the

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most simple methyl *p*-nitrophenyl esters and amides of benzoyl-Arg (Bz-Arg)[1] [46–48]. However, these substrates are rarely used now because of inherent problems; they lack sensitivity, have low solubility, and sometimes spontaneously hydrolyze (in the case of *p*-nitrophenyl esters).

The most popular cysteine peptidase substrates are the short chromogenic peptides Z-Phe-Arg-pNA (where pNA = *p*-nitroanilide), Z-Arg-Arg-pNA [17,49–52], and their fluorogenic analogs Z-Phe-Arg-AMC (where AMC = 4-amino-7-methylcoumaride), Z-Phe-Arg-AFC (where AFC = 4-amino-7-trifluoromethyl-coumaride), Z-Arg-Arg-AMC, and Z-Arg-Arg-AFC [50,53–56]. These compounds are moderately soluble and highly stable in water-based solvents, and they have kinetic characteristics desirable in peptidase assays. With these substrates, the values of K_m for the model enzyme papain vary from 0.08 to 32 mM and the values of k_{cat} vary from 0.2 to 34 s^{-1} [50]. However, Arg- and Lys-containing substrates are also hydrolyzed by trypsin-like serine peptidases; therefore, these substrates are not selective toward C1 cysteine peptidases. In addition, the production of arginine-containing substrates by a complex multistage chemical synthesis is complicated [56].

Previously, we enzymatically synthesized the selective chromogenic peptide substrate Glp-Phe-Leu-pNA (where Glp = pyroglutamyl) [57]. This substrate is specific for papain-like cysteine peptidases and was successfully used in the purification and evaluation of cysteine peptidases of various origins and levels of purity [38,57]. However, the use of this substrate is limited by its low solubility in water and the need to use high concentrations of organic solvents (20% dimethylformamide or dimethyl sulfoxide). To avoid these problems, we found that chromogenic and fluorogenic analogues of this substrate, containing an Ala residue in the P1 position, were better substrates for cysteine peptidases [58,59], and one of them, Glp-Phe-Ala-pNA, was successfully used to characterize complexes of digestive peptidases from insects [43–45,60].

In the current article, we have broadened the set of substrates containing the Ala residue in the P_1 position, modified the techniques of synthesis, and demonstrated the selectivity of the substrates for the characterization of C1 cysteine peptidases either in a homogeneous state or as part of complex multicomponent mixtures of digestive enzymes in the stored products pests *Tenebrio molitor* and *Tribolium* spp. (Coleoptera: Tenebrionidae).

Materials and methods

Enzymes

Papain (EC 3.4.22.2), stem bromelain (EC 3.4.22.32), ficin (EC 3.4.22.3), cathepsin B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15), bovine trypsin (EC 3.4.21.4), and subtilisin Carlsberg (EC 3.4.21.62) were obtained from Sigma–Aldrich (USA). Thermolysin (EC 3.4.24.27) and α-chymotrypsin (EC 3.4.21.1) were obtained from Fluka (Switzerland). Porcine pepsin (EC 3.4.23.1) was purified as described previously [61].

Chemicals

Acetonitrile (MeCN) for high-performance liquid chromatography (HPLC), special purity grade containing not more than 0.01% of water, was obtained from Lekbiofarm (Russia). Dimethylformamide (DMF) and triethylamine (TEA) of analytical grade (Reakhim, Russia) were further purified by the method in Ref. [62]. Trifluoroacetic acid (TFA) of analytical grade was obtained from Fluka. Acetic acid of analytical grade was obtained from Reakhim. Ethylenediaminetetraacetic acid (EDTA), calcium chloride, and dithiothreitol (DTT) were obtained from Sigma–Aldrich. Sephadex G-100 was obtained from Pharmacia (Sweden). L-Alanine 7-amido-4-(trifluoromethyl)coumarin trifluoroacetate (L-Ala-AFC × TFA), L-alanine 7-amido-4-methylcoumarin trifluoroacetate (L-Ala-AMC × TFA), L-alanine *p*-nitroanilide (L-Ala-pNA), and chromogenic substrates Bz-Arg-pNA, Z-Arg-Arg-pNA, and Z-Phe-Arg-pNA were obtained from Bachem (Switzerland). Derivatives of other amino acids and peptides were synthesized in our laboratory by standard techniques [63].

Synthesis of substrates by enzymes in solution

To begin the synthesis of *Glp-Phe-Ala-pNA*, 1.5 mg of α-chymotrypsin was added to a solution containing 112 mg (0.53 mmol) of L-Ala-pNA and 154 mg (0.53 mmol) of pyroglutamyl-phenylalanine methyl ester (Glp-Phe-OCH_3) [59] in 0.53 ml of DMF and 3 ml of 0.2 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.9). After 5 min, a voluminous precipitate was formed. The mixture was stirred for 1 h at 20 °C and left for 10 h at 0 °C. The precipitate was centrifuged, washed with 5% citric acid and water, and dried over NaOH in a vacuum.

Synthesis with subtilisin Carlsberg was performed as described above except that 0.2 M Tris–HCl buffer (pH 8.2) was used instead of the sodium carbonate buffer.

Glp-Phe-Ala-AFC was synthesized by the procedure similar to that described above for Glp-Phe-Ala-pNA, using 29 mg (0.1 mmol) Glp-PheOMe and 41 mg (0.1 mmol) CF_3COOH·Ala-AFC. All other enzymes and buffers were the same.

Synthesis of substrates by immobilized enzymes

Alternatively, substrates Glp-Phe-Ala-pNA (I), Glp-Val-Ala-pNA (II), Glp-Phe-Ala-AMC (III), and Glp-Phe-Ala-AFC (IV) were synthesized using immobilized chymotrypsin and subtilisin. Preparations of immobilized enzymes on poly(vinyl alcohol)–cryogel carrier were obtained according to the procedure in Ref. [64]. All substrates (I–IV) were obtained in analytical quantities in an anhydrous medium of polar organic solvents (DMF–MeCN, 20:80, vol%) in one cycle for 24 h according to the previously described method [59] using Glp-X-OMe (where X = Phe or Val), and Ala-Y (where Y = pNA, AMC, or AFC) as starting compounds. Glp-Phe-Ala-pNA (I) and Glp-Phe-Ala-AMC (III) were also synthesized during six and three consequent cycles of peptide production, respectively, according to the procedure described in Ref. [64]. After each cycle, the beads of biocatalyst were rinsed twice with the solvent mixture. Glp-Phe-Val-pNA and Glp-Phe-Ala-AFC were also obtained in preparative quantities as described previously [59]. The physic-chemical characteristics of substrates are presented in Table S1 of the online Supplementary material.

Analyses of synthesized compounds

Thin-layer chromatography (TLC) was performed on an Eastman Chromogram Sheet (Kodak, USA) in the following systems: (A) *n*-butanol–pyridine–water–acetic acid (15:12:10:3); (B) acetone–benzene–acetic acid (100:25:4). Compounds with a free α-amino group were detected with ninhydrin reagent, and peptides were visualized by spraying chlorinated plates with 0.05 M KI and exposure to ultraviolet (UV) light (254 and 290 nm) using UV analysis lamps (HP-UVIS, Sweden).

[1] *Abbreviations used:* Bz, benzoyl; pNA, *p*-nitroanilide; AMC, 4-amino-7-methylcoumaride; AFC, 4-amino-7-trifluoromethyl-coumaride; Glp, pyroglutamyl; MeCN, acetonitrile; HPLC, high-performance liquid chromatography; DMF, dimethylformamide; TEA, triethylamine; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; UV, ultraviolet; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; 3D, three-dimensional; UB, universal buffer; E-64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane; Abz, *o*-aminobenzoyl; CHTR, α-chymotrypsin; SL, subtilisin Carlsberg.