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Spontaneous, non-enzymatic breakdown of peptides during enzymatic protein hydrolysis

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ABSTRACT

It is expected that during the hydrolysis of proteins with specific enzymes only peptides are formed that result from hydrolysis of the specific cleavage sites (i.e. specific peptides). It is, however, quite common to find a-specific peptides (i.e. resulting from a-specific cleavage), which are often ignored, or explained by impurities in the enzyme preparation. In recent work in a whey protein isolate (WPI) hydrolysate obtained with the specific *Bacillus licheniformis* protease (BLP), 13 peptides of 77 identified were found to be the result of a-specific cleavage. These were formed after degradation of 6 specific peptides, after 5 different types of amino acids. The fact that other peptides were not hydrolyzed after these 5 amino acids suggests that the cleavages were not the result of a contamination with a different enzyme. In other systems, certain peptide sequences have been described to degrade chemically, under relatively mild conditions. This process is referred to as spontaneous cleavage. To test if the a-specific peptides observed in the WPI hydrolysis are the results of spontaneous cleavages, the parental peptides were synthesized. Surprisingly, 4 of the 5 synthesized peptides were indeed spontaneously cleaved under the mild conditions used in this study (i.e. 40 °C and pH 8) showing that peptides are less stable than typically considered. The rate of cleavage on the a-specific bonds was found to be enhanced in the presence of BLP. This suggests that the formation of a-specific peptides is not due to side activity but rather an enhancement of intrinsic instability of the peptides.

1. Introduction

Proteases are typically identified as specific or a-specific enzymes, depending on the number of different amino acids that function as a cleavage site. Specific proteases only hydrolyze peptide bonds next to one or two types of amino acid. The hydrolysis can take place either on the N-terminal (e.g. N-Lys protease), or C-terminal side (e.g. C-Lys protease) of the amino acid. A well-known example of a specific protease is trypsin, which is considered to be specific for Lys and Arg. Nevertheless, in hydrolysates obtained from such specific proteases, a number of a-specific peptides have been annotated. For instance, during hydrolysis by trypsin cleavages after Tyr, Trp and Phe are identified e.g. at pH 7.5 [1] or pH 8 [2]. Even in proteomics studies in which sequence grade trypsin was used, such a-specific cleavages have been annotated [3]. A-specific cleavage products were also identified in hydrolysates from BLP (*Bacillus licheniformis* protease), which is specific for Glu and Asp residues [4]. The a-specific cleavages were found to occur after four different amino acids in β-casein: 52-Phe; 128-Thr; 188-Gln; and 192-Leu [5]. For both enzyme preparations, the a-specificity was attributed to residual activities present in the enzyme preparation. For trypsin hydrolysis, the residual side activity was assumed to be due to chymotrypsin, which is specific for large hydrophobic amino acids (Trp, Tyr, Phe). For BLP hydrolysis, the a-specific cleavages were attributed to the remaining Subtilisin, which has a preference for aromatic residues (Phe, Tyr and Trp) and Leu residues [6]. However, even using a purified BLP preparation, a-specific peptides were found in hydrolysates of whey protein isolate by BLP [7]. Surprisingly, the cleavages of a-specific cleavage bonds are only occurring on a limited number of cleavage sites [7]. If the cleavages were the result of hydrolysis by another enzyme present, more of these cleavages would be obtained. In the latter study the molar concentration of all peptides was followed as a function of DH. Based on the concentration of the peptides, it was shown that the a-specific cleavages occur for a limited number of peptides after different types of amino acids. The cleavages occurred at positions 59 (Gln-Lys) in peptide β-lg[56–62]; 80 (Ala-Val) in peptide β-lg[75–89]; 141 (Lys-Ala), 145 (Met-His) and 150 (Ser-Phe) in peptides β-lg[135–157], β-lg[138–157], β-lg[135–158] and β-lg[138–158]. Interestingly, in longer peptides containing the same peptide bond (i.e. [63–89] or [113–157]), the peptide bonds were not cleaved. Moreover, the same amino acids in other parts of the sequence, e.g. 138 (Lys-Ala), were not used as cleavage site. Based on this, it is unlikely that the cleavages are the result of

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side activity of the enzyme preparation or impurities. Rather, it is considered to be the result of a type of autolytic mechanism that only occurs in certain specific peptide sequences. Still, in enzymatic protein hydrolysates, it is difficult to clearly identify the formation of these a-specific peptides, since they are in a complex mixture of peptides.

Such so-called spontaneous cleavages have been observed in specific isolated cases, such as the vasoactive intestinal peptide, a peptide hormone consisting of 28 amino acid residues [8]. This peptide showed an intrinsic autolytic activity and was cleaved at a rate of $1.5 \cdot 10^{-5}\ s^{-1}$ (at neutral pH and 38 °C). The spontaneous cleavage was inhibited when the secondary structure of the peptide was changed by the addition of sodium dodecyl sulfate. Another well-known example is the group of proteins referred to as inteins. Inteins are a part of the sequence of specific proteins involved in protein or RNA splicing [9]. The inteins are released from the rest of the protein by two protein cleavage events, as a result of autocatalytic cleavage [10]. These examples show the spontaneous cleavage of peptides due to specific structural properties. Furthermore, slow cleavages of peptide bond Phe-Gly in peptide Phe-Phe-Phe-Gly have been observed in neutral water solutions at room temperature at a rate of $3 \cdot 10^{-9}\ s^{-1}$ (corresponding to a half-life of 7 years). These cleavages were shown to be the result of uncatalyzed attack of the water and not due to impurities [11,12].

Another class of spontaneous cleavage is attributed to the presence of specific amino acids (asparagine, or serine) in a peptide sequence. The bonds after Asn can be hydrolyzed after an intramolecular nucleophilic attack, following the deprotonation of the amide group of the side chain of the aspartic acid [13]. The rate of this reaction is $1.6 \cdot 10^{-7}\ s^{-1}$ at pH 8 in MES buffer and 37 °C. Furthermore, the cleavage of peptide bonds on the N-terminal side of serine residues is considered to be the result of an intramolecular attack involving the hydroxyl group of the serine residue [14,15]. This reaction occurs at 37 °C and neutral pH at a rate of $1.7 \cdot 10^{-7}\ \mu mol \cdot s^{-1}$ (equivalent to 25% conversion after 500 h) and is 10 times faster at 60 °C.

From the above it becomes clear that not all peptides are intrinsically stable. On the contrary, several peptides are known to undergo spontaneous cleavage. The reported reaction rates of the spontaneous cleavage are not very high, but this phenomenon may still occur during enzymatic hydrolysis of proteins. If so, the a-specific cleavages observed even with very pure and specific enzymes may be due to such spontaneous cleavage. However, the phenomenon is not typically considered to occur during enzymatic hydrolysis of proteins.

In the hydrolysates obtained from WPI and BLP a number of a-specific peptides were annotated, and identified to be formed from certain parental peptides [7]. To identify the phenomenon and further study the a-specific cleavages of certain peptide bonds, five assumed parental peptides were synthesized. Firstly, the formation and kinetics of a-specific cleavage in the presence of the enzyme was determined to confirm the observations from the complex mixture [7]. In addition, the synthesized peptides were also incubated individually in the absence of enzyme, to test if there was indeed an intrinsic instability of the peptides.

2. Materials and methods

2.1. Materials

BLP (*B. licheniformis* protease — NS-37005) was obtained from Novozymes (Bagsvaerd, Denmark). The BLP (4.5% (w/w) protein, by the Dumas method N × 6.25; activity of 0.3 AU/mg/min as determined by the azocasein assay [16]) was partly insoluble and was fractionated as described before [17]. The freeze dried, water soluble material was found to contain 60% (w/w) protein (N × 6.25). The purity of the enzyme was determined from the UV peak area at 214 nm from RP-UPLC–MS to be 92%, but even 100% based on the peak area at 280 nm. In the chromatogram two main peaks were identified. The main peak (78%) was identified to be the enzyme BLP (23.6 kDa). A second smaller peak (14% of total UV_{214} area) was due to the pro-peptide (6.9 kDa) as described previously [17]. The enzyme has an activity of 3.9 AU/mg/min as determined by the azocasein assay. Bipro, a commercial whey protein isolate (WPI), was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). The WPI contained (by weight) 74.0% β-lactoglobulin, 12.5% α-lactalbumin, 5.5% bovine serum albumin and 5.5% immunoglobulin, according to specifications of the supplier. The protein content (N × 6.32) [18] of the powder was 93.4% (w/w) as determined by the Dumas method. Peptides β-lg[135–157] with the sequence KFDKALKALPMHIRLSFNPTQLE and β-lg[138–157] with the sequence KALKALPMHIRLSFNPTQLE were synthesized by Biomatik (Wilmington, DE, USA) and stored at −20 °C. The peptide sequences were identified using the MS signal and the identification was confirmed using MS/MS. Additionally the purity was determined using UV_{214} (Table 1). Three other peptides (described below) were synthesized in-house, using Fmoc-protected amino acids and Fmoc-Glu(OtBu) Wang resin (0.51 mmol/g loading), which were purchased from Novabiochem. HBTU, DIEA, Fmoc-Asp(OtBu) Wang resin (0.4–0.9 mmol/g loading), piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIS), N,N-dimethylformamide (DMF), β-lactoglobulin (purity > 90%) and all other chemicals were of analytical grade and purchased from Sigma.

2.2. Enzymatic protein hydrolysis

Whey protein isolate hydrolysates were performed as described previously [18]. WPI powder was dispersed at a concentration of 45% (w/v) in Millipore water, followed by stirring overnight at 4 °C. Insoluble parts were removed by centrifugation (30 min, 4000 *g*, 20 °C) and the supernatant obtained (30% (w/v)) was diluted to 1 and 5% (w/v) as determined by UV_{280} [18]. Hydrolyses of whey protein solutions were performed using a pH-stat at pH 8. Solutions of WPI at 1 and 5% (w/v) were hydrolyzed at pH 8 and 40 °C using NaOH 0.2 and 1 M respectively to keep the pH constant. Protein solutions (10 mL) were equilibrated for at least 15 min at 40 °C and adjusted to pH 8.0 before the addition of BLP dissolved at 5% (w/v) in Millipore water (0.30 μL of enzyme·$mg^{-1}_{protein}$) [7]. The degree of hydrolysis (DH) was calculated on the basis of the added volume of NaOH V_b at a concentration N_b, with α, the average degree of dissociation of α-NH groups ($1/\alpha = 1.20$ at 40 °C and pH 8.0), and h_{tot}, the total number of peptide bonds per gram protein substrate (h_{tot}(WPI) = 8.5) using Eq. (1)

$$DH(\%) = V_b \times N_b \times \frac{1}{\alpha} \times \frac{1}{M_p} \times \frac{1}{h_{tot}} \times 100. \quad (1)$$

Samples were taken during hydrolysis at different DH values and analyzed using UPLC–MS as described in the section below. Peptides from β-lactoglobulin were annotated in all samples by using the MS and MS/MS data and quantified using the UV data as described below.

2.3. Synthesis of the peptides

Peptides β-lg[56–62] with the sequence ILLQKWE, β-lg[75–89] KTKIPAVFKIDALNE and β-lg[75–85] KTKIPAVFKID were synthesized at Parma University, Italy, using Fmoc-SPPS on Wang resins preloaded with the C-terminal amino acids. The syntheses were carried out using an automated peptide synthesizer (Syro I, Biotage, Uppsala, Sweden). Amino acid coupling was performed in the presence of 5 equiv. of amino acid, 10 equiv. of DIEA and 4.7 equiv. of HBTU to the initial loading of the resin. Fmoc-deprotection was achieved by treatment of the resin with 40% (v/v) piperidine in DMF. After completion of peptide syntheses, the peptide-resins were dried under vacuum; cleavage of the peptides from the resins was achieved by treatment with a mixture of TFA, TIS and water (95:2.5:2.5) for 2 h at room temperature. The resins were removed by filtration and washed with TFA. The combined filtrates were then dried under N_2 flux. Cold ethyl ether (5 °C) was added to the residues to precipitate the unprotected peptides.