Synergistic Action of an X-Prolyl Dipeptidyl Aminopeptidase and a Non-Specific Aminopeptidase in Protein Hydrolysis

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Non-specific monoaminopeptidase (AP; E.C. 3.4.11) and X-prolyl dipeptidyl aminopeptidase (X-PDAP; E.C. 3.4.14.5), both from *Aspergillus oryzae*, demonstrate strong synergism in hydrolyzing proline-containing peptides. Incubation of AP alone with the peptide Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe does not generate free amino acids. However, when AP and X-PDAP are added in combination, complete and immediate hydrolysis of all peptide bonds, other than X-Pro bonds, is observed. In the enzymatic hydrolysis of casein, soy, and gluten, degree of hydrolysis (DH) values of 54, 54, and 47% were achieved, respectively, when subtilisin (E.C. 3.4.21.62) was supplemented with AP. Addition of a third enzyme, X-PDAP, resulted in significantly higher DH values of 69, 72, and 64%, respectively, establishing the utility of this synergism in protein hydrolysis.

Keywords: *Debittering; protein hydrolysate; aminopeptidase; proline dipeptidase; synergism; degree of hydrolysis*

INTRODUCTION

Flavor is one of the primary determinants used in our food choices. To have a wide flavor spectrum for different types of meat, marine, dairy, and other food products, the food industry has been using a large variety of taste-enhancing ingredients, including protein hydrolysates (*1*). The attractiveness of using enzymes in the production of protein hydrolysates is obvious. Enzymes are not only "natural," they have been utilized for centuries and are thus well accepted.

Short peptides, and especially free amino acids, serve positive roles in flavor formation by eliciting characteristic tastes of food (*2*). Long peptides, in general, are bitter (*3*−*6*), and thus extensive hydrolysis of proteins has a debittering effect as well. Among the essential amino acids, proline and glycine occupy a unique position because of their unusual structures: proline is cyclic and glycine is achiral. These peculiarities make these two particular residues resistant to enzymatic hydrolysis by exopeptidases, and oftentimes, limit the depth of hydrolysis that can be achieved. Ironically, although glycine and proline are problematic for enzymatic hydrolysis, they are important residues to liberate because they possess strong flavor.

Recently we have succeeded in discovering two secreted aminopeptidases in *Aspergillus oryzae*. The monoaminopeptidase has been shown to be a nonspecific enzyme with the strong capability of liberating N-terminal glycine and proline with high efficacy (*7*). In contrast, the dipeptidyl aminopeptidase has been found to be exceptionally reactive toward substrates that have N-terminal X-Pro sequences (*8*). In the present work, the synergistic effect of these two enzymes in the hydrolysis of a model peptide and its utility in boosting the degree of hydrolysis (*9*) of casein, soy, and gluten is described.

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MATERIALS AND METHODS

Chemicals. Chemicals used as buffers and reagents were commercial products of at least reagent grade. *para*-Nitroanilide substrates and the peptide Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe (that is Ala-Pro-Gly-[Ile^3,Val^5]-Angiotensin II) were from Sigma Chemical Co. (St. Louis, MO) or BACHEM California (Torrance, CA). Subtilisin (Alcalase 2.4L) was obtained from Novo Nordisk A/S (Bagsværd, Denmark). The sample contains 2.4 units/g (one unit will hydrolyze casein to produce 1.0 μmole of tyrosine per min at pH 7.5 at 37 °C). The recombinant AP from *A. oryzae* was expressed and purified as previously described (*7*).

Recombinant X-Prolyl Dipeptidyl Aminopeptidase. The nucleic acid sequence of the full length X-PDAP gene from *A. oryzae* (*8*) was inserted into an expression cassette for transformation into *Fusarium venenatum* (*7*). Assays were performed on the initial transformants. The highest yielding strain was grown in shake flasks. On day 5, the culture broths were filtered through Miracloth then subsequently frozen. The supernatant (20 mL) was thawed, centrifuged, and filtered through a 0.45-μm filter. The sample was desalted via ultrafiltration using a PM 10 ultrafiltration membrane. The conductivity of the sample was reduced to 2.5 mS using 20 mM sodium phosphate buffer, pH 7.0. The sample was loaded onto a Q-Sepharose Big Beads (Pharmacia, Piscataway, NJ) column (60 mL), which had been preequilibrated with 20 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient from 0 to 0.4 M NaCl in 20 mM sodium phosphate buffer, pH 7.0, over 10 column volumes. Fractions were assayed with Ala-Pro-pNa, as described below, and the active fractions were analyzed by SDS-PAGE. An enzyme sample of greater than 95% purity was obtained.

Enzyme Assays. Aminopeptidase activity was determined as previously described (*7*). The X-Prolyl dipeptidyl aminopeptidase activity was also previously described (*8*). 1 Unit of activity is defined as 1 μmole of *p*-nitroaniline liberated/min.

Hydrolysis of a Synthetic Peptide. The peptide Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe (APGDRIYVHPF) was dissolved in 50 mM sodium phosphate pH 7.5 buffer to a final concentration of 1 mg/mL. Each incubation mixture (1 mL) contained the peptide (0.86 mg) and either 0.1 units of AP, 0.1 unit of X-PDAP, or 0.1 unit of AP plus 0.1 unit of X-PDAP.

10.1021/jf001091m CCC: $20.00
Published on Web 03/15/2001

The reaction was performed at ambient temperature. At 0, 0.5, 1, 3, and 6 h, 150-μL aliquots of each reaction mixture were added to 150 μL of 0.1 N HCl and subsequently frozen. The derivatization (*10*) of 50 μL of the obtained solution was performed in total volume of 300 μL. The resulting free amino acids (20-μL injection volume) were analyzed by reversed-phase HPLC on a Hewlett-Packard series 1050 HPLC system (Palo Alto, CA) utilizing a C18 column from Vydac (Hesperia, CA).

Hydrolysis of Casein, Soy, and Gluten. The casein solution was prepared by dissolving 2.43 g of casein (91.2% protein, Novo Nordisk A/S, Bagsværd, Denmark) in 87.5 mL of distilled water at 50 °C for 1.5 h. After the pH was adjusted to 7.0 using 0.1 N NaOH, distilled water was added to achieve a final volume of 100 mL. The soy and gluten solutions were composed of 2.22% protein (Novo Nordisk A/S, Bagsværd, Denmark) in water at pH 7.0.

To each reaction vial, enzymes were added to either 9 mL of the casein solution or 9 mL of 2.22% soy or gluten. Distilled water was used to achieve a final volume of 10 mL. The reactions were performed at 50 °C for 18 h with constant stirring. The resulting products were incubated at 85−90 °C for 6 min to stop the enzymatic reactions. The degree of hydrolysis was determined using the method developed earlier (*11*) but ortho-phthalaldehyde was used instead of trinitrobenzenesulfonic acid.

Dosage Dependence of the Degree of Hydrolysis on X-PDAP Concentration. A standard reaction mixture (10 mL) was composed of 2% gluten, 4 units of AP, and 0.008 units of Subtilisin (Alcalase 2.4L). X-PDAP was then dosed into each reaction vial at concentrations ranging from 0.05 to 1.6 units. The incubation conditions and DH analysis method were the same as described above.

RESULTS AND DISCUSSION

The significance of deep enzymatic hydrolysis of proteins for flavor formation was realized decades ago. However, little progress has been achieved in this area, mainly because of the unavailability of enzymes with the necessary specificities. Recently, the potential of X-prolyl dipeptidyl aminopeptidase in the enhancement of enzymatic degradation of some casein-derived peptides has been demonstrated (*12*). Because of the lack of nonspecific exopeptidases, the attention has been primarily focused on aminopeptidases specific for hydrophobic amino acids. These types of enzymes have been shown to have some effect in reducing bitterness, such as the aminopeptidase from *Aeromonas caviae* (*13*). The major objective of this research was to demonstrate the great potential of proteases/peptidases, applied in the correct combination, in obtaining a high degree of hydrolysis of common food proteins such as casein, gluten, and soy.

The peptide APGDRIYVHPF was selected as a model mainly because of the Ala-Pro present at the N-terminus, but also for its diverse composition of amino acids. Because of its amino acid sequence, this peptide is resistant to hydrolysis by aminopeptidases. Possessing a uniquely broad substrate specificity, AP is able to liberate both N-terminal proline and glycine. It, nevertheless, does not demonstrate X-Pro aminopeptidase activity (*7*). As a result, when added alone, this enzyme is unable to overcome the Ala-Pro bond, and thus cannot access the post Ala-Pro amino acids. On the other hand, although X-PDAP is able to liberate the Ala-Pro dipeptide, it is unable to proceed further because its specificity is limited to X-Pro and X-Ala dipeptides (*8*). As expected, incubation of this peptide with either AP or X-PDAP alone revealed no release of free amino acids. In the incubation of this decapeptide with X-PDAP, an unknown peak was observed on the chromatogram in the later time points (data not shown). This peak most likely corresponds to the dipeptide Ala-Pro. When the enzymes were dosed in combination, considerable levels of glycine, aspartate, arginine, isoleucine, and tyrosine were observed at the first time point, 0.5 h (Table 1). Following a 3 h incubation, the detection of 0.89 nmoles of phenlyalanine (complete hydrolysis would yield 1.13 nmoles) indicated almost a complete hydrolysis of all peptide bonds in the peptide, other than X-Pro. Alanine, histidine, and proline were not detected in any of the reaction mixtures.

Table 1. Time Course of Hydrolysis on Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe (1.13 nmoles) Catalyzed by Aminopeptidase and X-Prolyl Dipeptidyl Aminopeptidase

released amino acid	amount of released amino acid (nmol) after incubation period				
	0 hr	0.5 hr	1.0 hr	3.0 hr	6.0 hr
Gly	0	0.58	0.87	1.08	0.90
Asp	0	0.47	0.70	1.11	0.96
Arg	0	0.47	0.70	0.83	0.81
Ile	0	0.45	0.66	0.79	0.79
Tyr	0	0.44	*a*	*a*	*a*
Val	0	0.10	0.28	0.89	1.02
Phe	0	0.02	0.26	0.89	0.97

[a] The quantity of tyrosine could not be accurately determined because of a coeluting peak.

Table 2. Performance of the Non-specific Aminopeptidase and X-Prolyl Dipeptidyl Aminopeptidase from *Aspergillus oryzae* in Proteolysis.

	degree of hydrolysis (%)		
	casein	soy	gluten
subtilisin (0.008 units)	17.5	23.5	15
subtilisin (0.008 units) + AP (1 unit)	48	45	43
subtilisin (0.008 units) + AP (1 unit) + X-PDAP (1 unit)	61	54	58
subtilisin (0.008 units) + AP (4 units)	54	54	47
subtilisin (0.008 units) + AP (4 units) + X-PDAP (1 unit)	68.5	71.5	64
subtilisin (0.008 units) + X-PDAP (1 unit)	18	24	15.5

There are no endoproteases that may, alone, give a high degree of hydrolysis of proteins. From the other side, one absolutely nonspecific exopeptidase, aminopeptidase, or carboxypeptidase, will, theoretically, hydrolyze a protein completely. However, in practice, native proteins are resistant to hydrolysis by exopeptidases. It is also clear that an endoprotease and an exopeptidase together will provide much more favorable kinetics. AP is almost completely nonspecific; it is clearly illustrated in our data for the hydrolysis of the peptide APGDRIYVHPF. It is thus not surprising that supplementing subtilisin with AP even at the low, 1-unit, level led to very high − 30.5%, 21.5%, and 28% − increases in DH for casein, soy, and gluten, respectively (Table 2). However, as one can see, a 4-fold higher dosage of AP, together with the same concentration of subtilisin, procured only 6%, 9%, and 4% added increases in DH, respectively. Taking into account the results shown above for the synthetic peptide, this effect of saturation can be rationally attributed to the proline residues serving as "road blocks" on the way of protein hydrolysis. [Casein, soy, and gluten contain different amounts of proline (11.57, 5.32, and 14.19%, respectively) in the amino acid compositions (*9*)]. If this theory were true,

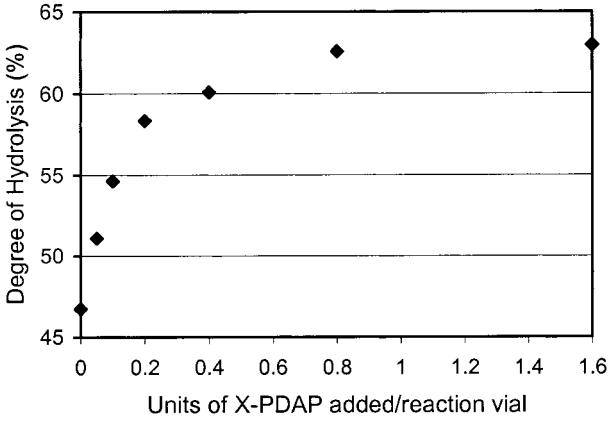

Figure 1. Dosage Dependence of DH of gluten on X-PDAP concentration. For more detailed information see the Materials and Methods subsection titled "Dosage Dependence of the Degree of Hydrolysis on X-PDAP Concentration".

the supplementation with X-PDAP should result in a significant increase in DH. Upon the addition of X-PDAP in combination with subtilisin and AP, the DH values rose by 14.5, 17.5, and 17%, respectively (Table 2). As a result, very high values of DH were achieved: 68.5% for casein, 71.5% for soy, and 64% for gluten.

As might be expected, a high dosage of X-PDAP is not obligatory. Significant increases in DH were observed at relatively low levels of X-PDAP, with saturation being achieved at 0.8 units (Figure 1). It was also seen that supplementing subtilisin with only X-PDAP had practically no effect on DH values (Table 2). These results clearly demonstrate the synergistic, and not additive, role of X-PDAP in a protein hydrolysis.

The results obtained in this study provide strong support for the idea of a replacement of chemical hydrolysis of proteins with an enzymatic competitor at the industrial level. A three-enzyme system consisting of a serine endoprotease, together with a nonspecific aminopeptidase and an X-prolyl dipeptidyl aminopeptidase, has been shown to be sufficient to achieve exceptionally high levels of DH. The origin of the enzymes that have been used in this study should be stressed separately. Subtilisin is one of the most commonly used industrial proteases. Both the nonspecific aminopeptidase and X-prolyl dipeptidyl aminopeptidase have been cloned from *Aspergillus oryzae* and expressed recombinantly (*7*, *8*). Advances in recombinant technology of the fungus *A. oryzae* allow for the possibility of this three-enzyme system to be commercially viable for achieving high DH.

ABBREVIATIONS USED

AP, nonspecific monoaminopeptidase; X-PDAP, X-prolyl dipeptidyl aminopeptidase; DH, degree of hydrolysis.

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Received for review September 1, 2000. Revised manuscript received February 6, 2001. Accepted February 7, 2001.

JF001091M