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Protease-catalysed condensation–oligomerisation of hydrophobic peptides as a means of flavour modification

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Abstract

Hydrophobic peptides such as Val–Leu, Gly–Leu and Ser–Leu can undergo a protease-catalysed condensation reaction in water or aqueous ethanol. The enzyme catalyses an equilibrium between peptide bond formation and breakage, and the reaction appears to be driven towards condensation by precipitation of the products. Electrospray mass spectroscopy (ESMS) of the products showed them to be oligomers of the starting peptide from the dimer up to as far as the heptamer, the tetramer generally being the major product. If the starting dipeptide is bitter, the oligomers are much less so. Monitoring of the reaction was undertaken using ^{13}C-NMR analysis of 1 $-$ ^{13}C-labelled marker peptides. This allowed precise determination of the extent of reaction under various conditions. This nuclear magnetic resonance (NMR) technique may be applicable to the study of peptide bond rearrangement in a variety of complex mixtures.

Keywords: Protease; Protein hydrolysate; Peptide; Bitterness; Transpeptidation; Hydrophobic

1. Introduction

The formation of hydrophobic bitter peptides during proteolysis of proteins, such as casein or soy isolate, is well known and is a serious problem which limits the use of protein hydrolysates to specialty food products such as hypoallergenic infant formulas [1,2]. There are various ways to reduce the bitterness, including masking, hydrolysis of bitter peptides with exopeptidases, or extraction of bitter peptides using hydrophobic adsorbents or organic solvents, but each method has drawbacks [2].

During a study of the use of proteases to modify the structure of bitter peptides and thereby reduce their bitterness, we found a novel peptide condensation reaction that can occur even in water. The characteristics of this reaction are described here.

2. Experimental

2.1. Materials

Enzymes were obtained from commercial suppliers—Alcalase and Neutrase from Novo Nordisk (Denmark), Corolase from Rohm

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(Germany) and Papain from Sigma (USA). Unlabelled peptides were obtained from Sigma, and the labelled equivalents were synthesised by standard procedures from 1-^{13}C-labelled amino acids obtained from Icon Services (NJ, USA). The labelled peptides were synthesised with the labelled amino acid at their C-terminus.

2.2. Enzymic reaction method

Peptide substrates and enzyme were added to water or aqueous ethanol (1 ml total volume) and stirred continuously at 40°C for 24 h (a time period determined to be long enough for the reaction to reach equilibrium). For isotopic labelling experiments, substrates were an 85:15 mixture of unlabelled and labelled peptide. Reaction mixtures were boiled to inactivate the enzyme, freeze-dried, then analysed by solid state (if insoluble products were formed) or solution (NMR).

2.3. ^{13}C-NMR analysis

The ^{13}C-labelled C-terminal carboxyl group gives a large ^{13}C-NMR signal at around 180 ppm. Formation of a peptide bond from this carboxyl group causes the signal to shift to around 170 ppm (Scheme 1). Percentage conversions were determined from the relative intensities of the two signals. Labelled peptide was diluted 85:15 with unlabelled material for enzyme assays; this gave acceptable signal strength without excessive consumption of labelled material.

2.4. Taste testing

A modification of a literature procedure [3] was used. Samples were tasted as 5% w/v aqueous solutions and rated against a 6-point scale of quinine sulphate standards of concentration 17 (2), 34 (3), 51 (4), 68 (5) and 85 (6) mg/l. A rating of one indicated undetectable bitterness.

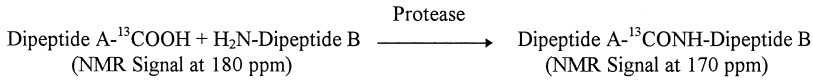
3. Results and discussion

Ethyl esters of bitter dipeptides are known not to be bitter [4], whereas hydrophobic oligopeptides generally have a very low water solubility; and any taste they have should not be readily perceived. Preliminary experiments were aimed at finding conditions under which proteases could convert model bitter peptides into either of these derivatives. The treatment of Val–Leu (chosen because it is bitter, commercially available and easy to make in labelled form) with Alcalase in 95% ethanol, 50% ethanol, or water resulted in formation of a precipitate.

The precipitate was insoluble in all solvents tested except formic acid (known to be a good solvent for hydrophobic oligopeptides) and thus, was not amenable to chromatographic analysis. Solid state NMR analysis, however, showed extensive (50–70%) conversion of labelled free carboxyl groups into peptide bonds and no sign of signals due to ethyl esters—a result which strongly suggested that oligomerisation had occurred.

3.1. Effect of oligomerisation on flavour

The bitterness scores of Val–Leu and the dried reaction mixtures from the three solvent systems were: Val–Leu: 6, 95% v/v ethanol: 3, water: 4, 50% v/v ethanol: 5. The precipitates from the three experiments were isolated by centrifugation and all scored as 2, whereas all of



Scheme 1. Principle of analysis.

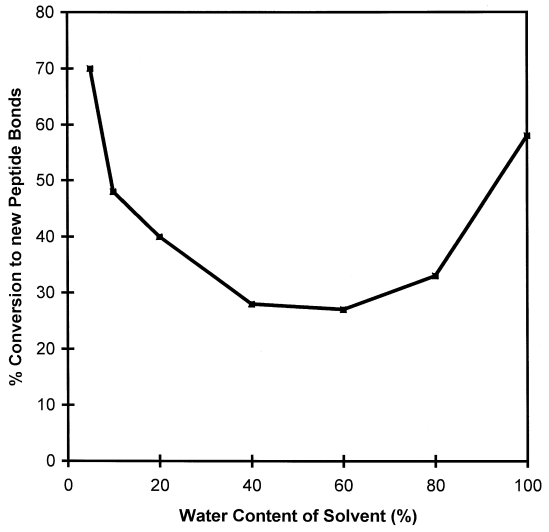


Fig. 1. Extent of oligomerisation (measured as peptide bond formation) of 1 – ^{13}C–Val–Leu as determined by solid sate NMR as a function of water content in aqueous ethanol.

the corresponding water soluble fractions were scored as 6.

3.2. Effect of varying reaction conditions

The reaction was repeated in water–ethanol mixtures of various compositions and proceeded, to some extent, under all conditions tested (Fig. 1). The reaction yield was less affected by substrate concentration than one might expect (peptide bond formation from Val–Leu in water was 24% at 0.5% w/v substrate and 60% at 20% w/v substrate).

Generally, conditions which favoured high peptide bond formation (e.g., 5% or 100% water, Fig. 1) gave larger amounts of precipitate and greater reductions in bitterness. These results indicate that the protease catalyses an equilibrium of peptide bond formation and breakage (i.e., transpeptidation) which is driven towards net bond formation by the products precipitating into a form which is not susceptible to enzymic hydrolysis. There can be no other driving force for a protease-catalysed net bond-forming reaction (which liberates water) in 100% aqueous solution. A precipitation-driven reaction would explain the 'U'-shaped profile of yield vs. water content and suggests that the products are most soluble in 60% ethanol (significant enzyme activity remained at the end of the reaction in all solvent mixtures).

3.3. Effect of different enzymes and dipeptide substrates

To determine the general applicability of the reaction, it was tried with other proteases and other peptides of varying hydrophobicity (Table 1). Clearly, of the ones tested, only the more hydrophobic peptides were converted by the enzymes into significant quantities of oligopeptide. The question remained as to why the more polar peptides did not give a precipitate, i.e., was there no reaction at all, or did the equilibrium of peptide bond formation and breakage give only soluble products that did not precipi-

Table 1
Conversion of carboxyl groups to peptide bonds (%)

Enzyme	Substrate (Q)				
	Gly–Gly (0)	Ala–Gly (365)	Val–Gly (845)	Leu–Gly (1210)	Val–Leu (2055)
Alcalase	< 1	< 1	1	25	57
Neutrase	4	< 1	< 1	39	56
Corolase PP	2	2	4	3	33
Papain	2	6	< 1	17	21

Each peptide substrate was a 15:85 mixture of labelled and unlabelled materials. Conversion is defined as the area of the peptide bond NMR signal expressed as a percentage of the combined areas of the peptide bond and free carboxyl signals. Quantities of enzyme used were: 50 μl Alcalase, 100 μl Neutrase or 5 mg Papain or Corolase (in 1 ml of 10% substrate solution). Q is a measure of hydrophobicity [4]; $Q = 1400$ is accepted as the lower limit for bitterness to be detectable.

Table 2
Effect of 'diluting' ^{13}C–Val–Leu with various dipeptides

'Diluting' peptide	% Conversion to peptide	% Water insoluble products
Gly–Gly	78	10.2
Ala–Gly	64	10.7
Val–Gly	74	10.7
Leu–Gly	78	17.3
Val–Leu	60 ± 7	59 ± 8

Val–Leu figures are the range obtained from several experiments. 15% ^{13}C–Val–Leu was the marker in all cases. Alcalase was used for all reactions. NB: Peptide bond breakage is not detected by NMR.

tate and accumulate. In another experiment therefore, labelled Val–Leu was used as a marker and added to itself and the other peptides (Table 2).

Although peptide bond formation is extensive with all five 'diluting' peptides, indicating that transpeptidation is extensive, precipitation only occurs, to a large extent, with Val–Leu itself as the diluent. This indicates that oligopeptides formed from labelled Val–Leu with other hydrophilic peptides are relatively water-soluble and do not precipitate and accumulate in the reaction. Oligopeptides formed from hydrophilic peptides alone would be even more water-soluble. Hence, hydrophilic peptides probably do take part in reactions but there is no precipitation effect to drive the net formation of peptide bonds.

3.4. Determination of the nature of the products

NMR analysis gave an accurate assessment of the extent of peptide bond formation but no indication of the size of the product oligopeptides. ESMS analysis was therefore applied to attempt to detect ions corresponding to larger species. This was successful (Table 3) and oligomers up to the heptamer were detected. Val–Leu yielded larger amounts of oligomer compared with other less hydrophobic dipeptides. No rearranged species were detected with the peptides shown, but Ser–Leu (Fig. 2) yielded oligomers and also showed ions corresponding to odd numbers of amino acid residues, the most intense being 732.3 (Leu_4Ser_3) and 932.9 (Leu_5Ser_4).

Table 3
Electrospray mass spectroscopy (ESMS) results

Substrate	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer
Gly–Val expected	174.2	330.4	486.6	642.8	799	955.2	1111.4
Gly–Val found	174.9	–	–	–	–	–	–
Gly–Leu expected	188.2	358.4	528.6	698.8	869	1039.2	1209.4
Gly–Leu found	189 (75)	358 (2)	529.4 (4)	699.7 (14.5)	869.6 (3.5)	–	–
Gly–Met expected	206.3	394.6	582.9	771.2	959.5	1147.8	1336.1
Gly–Met found	206.9 (75)	395.3 (2)	583.2 (4)	771.2 (8.5)	959.7 (5)	1147.8 (1.5)	1336 (0.3)
Gly–Tyr expected	238.2	458.4	678.6	898.8	1119	1339.2	1559.4
Gly–Tyr found	239 (95)	458 (1.5)	679 (1.1)	899.6 (1.7)	–	–	–
Val–Leu expected	230.3	442.6	654.9	867.2	1079.5	1291.8	1504.1
Val–Leu/EtOH Found	231.3 (1.4)	–	–	867.7 (74)	1080.3 (5)	–	–
Val–Leu/Water Found	231.6 (39)	445.5 (20)	655.4 (8)	867.6 (21)	–	–	–

Table shows masses of observed ions (amu) with expected masses of oligomers. Relative intensities (%) are shown in parentheses. All reactions were done with a substrate concentration of 10% w/v, using Alcalase. When the water-insoluble reaction products were isolated by centrifugation and analysed separately, they were found to contain mostly oligomers, whereas the water-soluble material was mostly starting dipeptide.

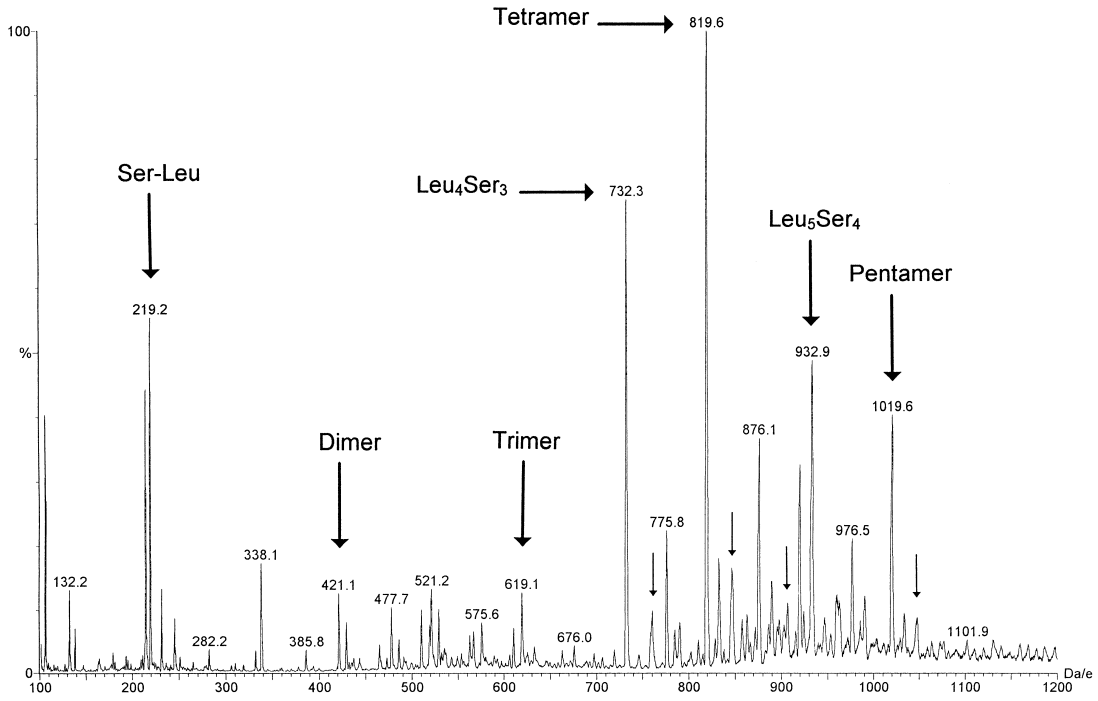


Fig. 2. ESMS spectrum of Ser–Leu treated with Alcalase in 95% ethanol. Small arrows indicate ions 28 amu heavier than other more intense ions. These may be due to formic acid adducts; formic acid was used to dissolve the otherwise insoluble samples for analysis.

Mixtures of two dipeptides (in the same proportions as the experiments in Table 2) treated with Alcalase in water were also analysed by ESMS. In some of these, various rearranged products were detected; in all cases, oligomerisation of the hydrophobic dipeptide was much reduced. For example, Val–Leu and Gly–Gly showed the following:

$$\text{Val–Gly}\,(174.3),\ (\text{Val–Leu})_3\,(655.2),\ \text{Val}_3 \times \text{Leu}_3\text{Gly}_2\,(769.3),\ (\text{Val–Leu})_4\,(867.7).$$

4. Conclusion

This study has demonstrated that dipeptides can be condensed by proteases into higher oligomers in solvent mixtures of various polarities. The reaction appears to be an equilibrium of bond formation and breakage which is driven towards net condensation by product precipitation. Although a condensation reaction in water might seem unlikely, recent studies on enzymic peptide synthesis have produced similar results [5,6]. Since the oligomerised peptides have a much reduced bitter taste, this approach may have applications in reducing the bitterness of hydrophobic peptides extracted from hydrolysates. This would allow the hydrophobic material to be added back to the hydrolysate, thus maintaining its amino acid balance. This possibility is currently under study.

When hydrophobic peptides are mixed with more polar peptides, the oligomerisation is partially inhibited and transpeptidation products can be detected. This indicates that rearrangement of peptides could be significant during production of protein hydrolysates, especially the accumulation of hydrophobic fragments into large oligopeptides. These might precipitate or fold into secondary structures that are resistant to

hydrolysis and lead to insoluble residues or species which will not pass through ultrafiltration membranes (used to control peptide size distributions for non-allergenic products). Studies are currently underway to determine how significant these processes are in large scale proteolysis and what could be done to minimise them.

The use of ^{13}C-enriched peptides allows the monitoring of bond formation and breakage in complex reaction mixtures without separation and purification. Even solid or semi-solid samples can be analysed by solid state NMR. This technique may have various applications in the monitoring of enzyme reactions in foods, especially in slow processes such as degradation during storage, cheese ripening etc. Provided the right labelled species is chosen, its behaviour can be taken as representative of similar species in the mixture.

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