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Specificity of two genetically related cell-envelope proteinases of *Lactococcus lactis* subsp. *cremoris* towards α_{s1}-casein-(1–23)-fragment

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The specificity of two genetically related cell-envelope serine proteinases (P_I-type and P_{III}-type) of *Lactococcus lactis* subsp. *cremoris* towards the α_{s1}-casein-(1–23)-fragment, an important intermediate product of primary chymosin-directed proteolysis in cheese, has been established. Both enzymes showed, at pH 6.5 and under relatively low-ionic-strength conditions, a characteristic, mutually different, cleavage pattern that seems, in the first instance, to be determined by the charge *N*-terminal to the cleaved bond. With P_I, three cleavage sites were found in the *N*-terminal positively charged part of the peptide and, with P_{III}, three sites were found in the *C*-terminal negatively charged part. Comparison of the specific cleavage sites in this peptide and those in β-casein revealed similarities with respect to the different residues which can occur *N*-terminally to the cleaved bond. The properties of these substrate residues match with the structural and various interactive features of the respective binding regions of the enzymes predicted on the basis of a close sequence similarity of the lactococcal proteinases with the subtilisin family. A hydrophobic interaction and/or hydrogen-bridge formation seems to govern the binding of the first amino acid residue *N*-terminal to the scissile bond. The more distantly *N*-terminally positioned sequence of residues apparently is attracted electrostatically by a negative charge in the binding region of P_I and by a positive charge in that of P_{III}, provided that the opposite charge is present at the appropriate position in this sequence. Hence a specific electrostatic binding may occur; additionally, hydrophobic interaction and/or hydrogen-bond formation is important.

INTRODUCTION

Two catalytically divergent cell-envelope-located serine proteinases (namely the P_I-type and the P_{III}-type) can be distinguished among strains of *Lactococcus lactis* subsp. *cremoris* (Visser *et al.*, 1986). These proteinases are essential to the organism for growth in media with protein as the sole source of nitrogen and essential amino acids. The enzymes have been purified and characterized (Exterkate & de Veer, 1987, 1989). The complete amino acid sequences have been deduced from the nucleotide sequences of the genes coding for the proteinases (Kok *et al.*, 1988; Vos *et al.*, 1989); these show > 98% sequence similarity. On the basis of sequence similarity with the subtilisin family, the amino acid residues of the active-centre and the substrate-binding region have been predicted (Vos *et al.*, 1989). The two lactococcal enzymes showed among themselves five amino acid differences in this region, two involving a change in charge (Vos *et al.*, 1989). The proteinases also show a different preference with respect to the caseins and a different specificity with respect to the cleavage of peptide bonds in these caseins (Visser *et al.*, 1986). A cation-binding site in the P_I-type proteinase, which is not present in the P_{III}-type, is mainly responsible for the difference between these enzymes with respect to the rate of conversion of small chromophoric peptides carrying a positive charge at the right position (Exterkate, 1990).

From the specificity of the P_I-type proteinase in its action on β-casein, it can be concluded that specific cation attraction does not contribute to a selective cleavage of peptide bonds in β-casein initially. The bonds split did not reveal a clear specificity with respect to residues close to these peptide bonds (Visser *et al.*, 1988; Monnet *et al.*, 1989).

Lactococci are used as starters in cheese making. They are essential for the acidification of the cheese milk and for the ripening process (namely proteolysis and flavour development) in Gouda cheese. Primary proteolysis of the caseins in cheese by the action of the cell-envelope proteinase initially is not significant compared with the contribution of chymosin (Exterkate, 1987). However, the proteinase is essential for the conversion of the products of chymosin action [salt-soluble nitrogen (SN) fraction] in cheese to amino acid nitrogen (Exterkate, 1987).

This conversion is related to flavour development in cheese, and therefore can be regarded as crucial for the complex process of ripening in general. The target of initial primary proteolysis by chymosin is α_{s1}-casein (α_{s1}-CN), and the main salt-soluble product of this action is α_{s1}-casein-(1–23)-fragment [α_{s1}-CN(f1–23)] (Exterkate, 1987). This peptide therefore seems an obvious substrate in order to gain insight in, on the one hand, the cleaving-specificity of cell-envelope proteinases towards (relatively large) peptides and, on the other hand, the initial crucial step of SN conversion in cheese.

MATERIALS AND METHODS

Preparation of crude α_{s1}-CN(f1–23)

Crude α_{s1}-CN(f1–23) was obtained by incubating an ($\alpha_{s1}+\beta$)-casein preparation with calf rennet and subsequent fractionation of the hydrolysate with $CaCl_2$. Full details of the procedure can be obtained from F.A.E. on request. The peptide was purified as described below.

Proteinase preparations

Lactococcus lactis subsp. *cremoris* strains HP and AM_1 were used as a source of P_I-type and P_{III}-type proteinase respectively. Pure proteinase preparations were obtained as described previously (Exterkate & de Veer, 1987, 1989). Dilutions were made in water immediately before use. The final dilution contained (per ml) an amount of enzyme which releases approx. 13000 (P_I-

Abbreviations used: α_{s1}-CN, α_{s1}-casein; α_{s1}-CN(f1–23), α_{s1}-casein-(1–23)-fragment; SN, salt-soluble nitrogen.

type) and 7500 c.p.m./min (P_{III}-type) from *methyl*-^{14}C-labelled β-casein (sp. radioactivity ~ 52000 c.p.m./mg) (Exterkate & de Veer, 1987).

Enzymic hydrolysis

To 100 μl of a solution of pure α_{s1}-CN(f1–23) in distilled water (8 mg/ml) was added 25 μl of the proteinase solution and 35 μl of 0.125 M-imidazole/acetic acid buffer, pH 6.5. The mixture was incubated at 30 °C and, at various times, aliquots (20 μl) were taken in order to monitor the reaction by h.p.l.c. The sample was heated for 10 min at 80 °C in order to inactivate the enzyme, and then freeze-dried.

For preparative purposes, reaction mixtures, incubated for 30 min, were treated accordingly.

Purification of the peptides

α_{s1}-CN(f1–23) and the derived peptides obtained by proteinase P_I and P_{III} action, were purified by reversed-phase h.p.l.c. using two M6000 A or M510 pumps (Waters Associates) controlled by an automatic gradient controller (Waters), a Gilson 231/401 or an ISS-100 (Perkin–Elmer) automatic sample injector, a model-481 u.v. detector (Waters), a Kratos 783 G u.v. detector, a Waters Maxima 820 data system, a reversed-phase C_{18} Cartridge (Bio-Rad) as the guard column, a HiPore RP-318 reversed-phase analytical (250 mm × 4.6 mm; Bio-Rad) and semi-preparative (250 mm × 10 mm; Bio-Rad) column. The columns were kept at 30 °C in a Kipp Analytica 9222 or a Waters TCM column oven. Solvent A consisted of acetonitrile/water/trifluoroacetic acid (100:900:1, by vol.); the composition of solvent B was acetonitrile/water/trifluoroacetic acid (900:100:0.7, by vol.).

The crude α_{s1}-CN(f1–23) was dissolved in solvent A (5 mg/ml). After centrifugation (13000 *g*; 10 min), 400 μl samples were applied to the preparative column, and elution was performed with a linear gradient from 20 to 35% B in A over a period of 25 min, followed by an increase to 70% B in A over the next 5 min. The flow rate was 3 ml/min. The α_{s1}-CN(f1–23) peak was collected using a LKB 2211 Superrac. The total fraction pooled over several runs was freeze-dried. The purity of α_{s1}-CN(f1–23) preparation was checked on the analytical column with an adjusted two-stage linear gradient (flow rate 0.8 ml/min), and also by amino acid analysis and end-group determination.

Hydrolysates of α_{s1}-CN(f1–23), obtained after 30 min of incubation with either P_I or P_{III}, were dissolved in water (10 mg/ml), diluted (1:1) with solvent A and filtered through a 0.22 μm-pore-size filter. Samples (120 μl) were applied to the preparative column, and fractions were collected during a five-step linear gradient elution (flow rate 3 ml/min) from 0 to 60% B in A over a total period of 68 min for the hydrolysate obtained by the action of P_I (that is, from 0 to 2% over the first 10 min, from 2 to 8% over the period 18–21.5 min, from 8 to 24% over the period 31.5–43 min, from 24 to 30% over the period 50–65 min, and from 30 to 60% over the last 3 min). A four-step linear gradient from 10 to 60% over a period of 52 min was applied for the hydrolysate produced by P_{III} (that is, from 10 to 14% over the first 5 min, from 14 to 23% over the period 17–33 min, from 23 to 32% over the period 38–47 min and from 32 to 60% over the period 47–52 min). The fractions were concentrated by evaporation in a Rotavapor RE 111 (Büchi) and then freeze-dried. The purity of the peptides was checked by re-chromatography carried out with the analytical column, applying the gradients used for the preparative runs and a flow rate of 0.8 ml/min.

For comparative purposes, peptides in the sample taken after different periods of incubation were separated with a one-stage linear gradient from 0 to 50% B in A over a period of 50 min. The freeze-dried samples were solubilized in solvent A (1 mg/ml), and 20 μl was injected.

Analysis of the peptides

Amino acid analysis was performed on an LKB type 4151 amino acid analyser after hydrolysis of the peptides with 6M-HCl in evacuated tubes at 110 °C for 24 h.

N-terminal-amino-acid identification was done by using the dansyl procedure (Gray, 1972). Dansyl-amino acids were separated on 5 cm × 5 cm polyamide layer sheets (Woods & Wang, 1967) and by reversed-phase h.p.l.c. (Wilkinson, 1978).

C-Terminal groups were detected by amino acid analysis after treatment with carboxypeptidase Y at pH 5.5 and 37 °C (Hayashi, 1977). Analytical results were matched with the known amino acid sequence of α_{s1}-CN(f1–23) (Mercier *et al.*, 1971).

RESULTS AND DISCUSSION

The course of α_{s1}-CN(f1–23) degradation by the action of the P_I- or P_{III}-type proteinase is shown in Figs. 1(*a*) and 1(*b*) respectively. In both cases the substrate was converted almost completely within 30 min. Each of the enzymes produced characteristic and completely different degradation patterns and showed only very limited production of components which were typical for the action of the other proteinase.

The products were isolated by h.p.l.c. in a preparative column

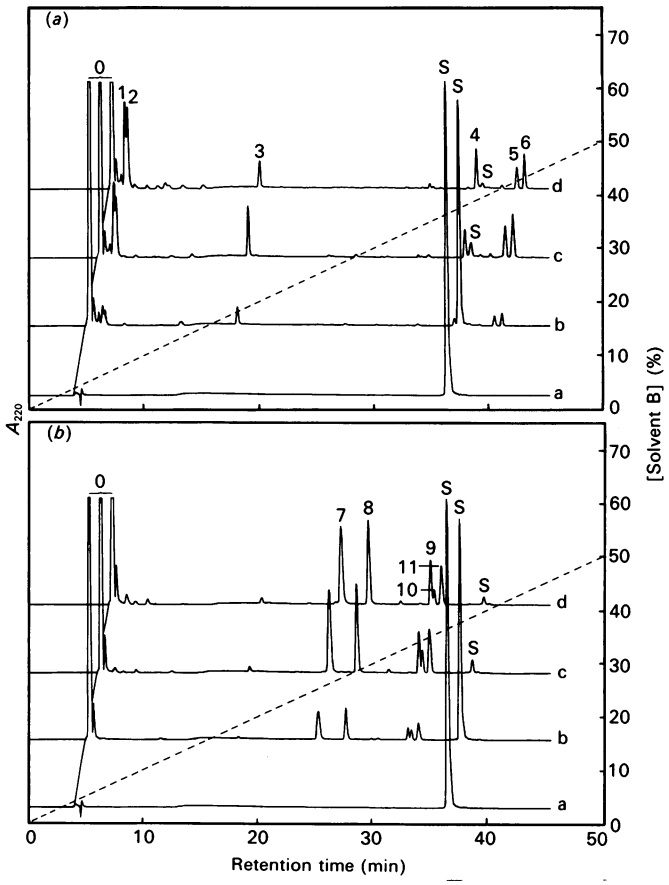

Fig. 1. Reversed-phase h.p.l.c. pattern of the products of 5 (pattern b), 30 (pattern c) and 60 (pattern d) min of incubation of α_1-CN(f1–23) with (*a*) the P_I-type or (*b*) the P_{III}-type proteinase (pH 6.5; 27 mM-imidazole/acetic acid buffer)

The products of P_I action (numbered 1–6) and those of P_{III} action (numbered 7–11) have been identified. The pure substrate α_{s1}-CN(f1–23) (S) is shown in pattern a. The patterns are shifted with regard to pattern a (for clarity); the retention-time axis relates only to pattern a. Fraction 0 (injection peak) contained 220 nm-absorbing material in the case of the incubation mixtures. The broken line represents the gradient applied.

Table 1. Amino acid composition (mol/mol) and end groups of the purified peptides 1–6 obtained by proteinase-P_I action and 7–11 obtained by proteinase-P_{III} action and isolated by semi-preparative h.p.l.c.

Values in parentheses indicate the number of residues expected on the basis of the composition of the proposed fragments.

	Proteinase ...	P_I							P_{III}				
Amino acid	Peptide ...	1	2	3	4	5	6	S*	7	8	9	10	11
Asx		0.1 (–)	0.1 (–)	0.2 (–)	2.1 (2)	2.1 (2)	2.0 (2)	2.1 (2)	1.1 (1)	0.1 (–)	1.8 (2)	1.1 (1)	2.1 (2)
Glx		1.3 (1)	0.3 (–)	2.2 (2)	2.1 (2)	4.0 (4)	3.0 (3)	4.0 (4)	3.0 (3)	3.0 (3)	0.9 (1)	1.2 (1)	4.0 (4)
Pro		2.1 (2)	1.8 (2)	3.0 (3)	–	1.7 (1)	1.8 (1)	3.3 (3)	2.6 (3)	3.1 (3)	0.2 (–)	0.2 (–)	3.5 (3)
Gly		0.2 (–)	0.2 (–)	1.3 (1)	0.1 (–)	1.1 (1)	1.1 (1)	1.1 (1)	1.1 (1)	1.1 (1)	0.1 (–)	0.1 (–)	1.1 (1)
Val		0.1 (–)	0.1 (–)	0.1 (–)	0.9 (1)	1.0 (1)	1.0 (1)	1.1 (1)	1.0 (1)	0.9 (1)	0.1 (–)	0.1 (–)	1.0 (1)
Ile		1.1 (1)	1.0 (1)	1.1 (1)	–	0.1 (–)	0.1 (–)	1.0 (1)	1.0 (1)	1.0 (1)	–	–	1.1 (1)
Leu		0.2 (–)	0.2 (–)	1.2 (1)	3.1 (3)	4.0 (4)	4.0 (4)	4.0 (4)	2.1 (2)	2.2 (2)	2.0 (2)	2.0 (2)	4.1 (4)
Phe		0.1 (–)	–	0.1 (–)	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)	–	–	1.0 (1)	1.0 (1)	0.1 (–)
His		1.8 (2)	1.9 (2)	1.7 (2)	–	0.1 (–)	0.1 (–)	1.9 (2)	1.9 (2)	1.9 (2)	–	–	1.9 (2)
Lys		2.0 (2)	2.0 (2)	1.9 (2)	0.1 (–)	0.2 (–)	0.2 (–)	2.0 (2)	2.0 (2)	2.0 (2)	0.1 (–)	0.1 (–)	2.0 (2)
Arg		1.1 (1)	1.0 (1)	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.9 (2)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
N-Term.† ...		Arg	Arg	Arg	N.R.‡	Glx	Gly	Arg	Arg	Arg	Asx	Glx	Arg
C-Term.§ ...		-(Ile, His, Lys)–Gln	-(Ile, His, Lys)	-(Gln, Gly)–Leu–Pro–Gln	-(Leu, Phe, Arg)	-(Leu, Phe, Arg)	-(Leu, Phe, Arg)	-(Leu, Phe, Arg)	-(Asn, Val, Leu)	-Glu–Val–Leu	-(Leu, Phe, Arg)	-(Leu, Phe, Arg)	-Glu–(Asn, Leu, Leu)
Proposed fragment‖ ...		1–9	1–8	1–13	14–23	9–23	10–23	1–23	1–17	1–16	17–23	18–23	1–21

* Substrate.
† Term. = terminus.
‡ No reaction, owing to *N*-terminal pyroglutamic acid formation.
§ Amino acids in parentheses: no attempts were made to establish the sequence in these cases.
‖ According to the amino acid sequence determined by Mercier *et al.* (1971).

using adjusted gradients (results not shown). The purity of the components in the collected effluents was established by re-chromatography in the analytical column. The purified products (numbered in Fig. 1) could be identified after amino acid analysis and end-group analysis (Table 1). Fig. 2 shows the amino acid sequence of the substrate α_{s1}-CN(f1–23) and the cleavage sites found for each enzyme. The peptides identified in this way account for cleavage of the bonds between residues 8 and 9, 9 and 10, and 13 and 14 for the P_I-type proteinase and between 16 and 17, 17 and 18, and 21 and 22 in case of the P_{III}-type proteinase. The kinetics of the reactions do not show a clear preference for one of the peptide bonds. P_I seems also able to cleave the latter bonds, although at a very low rate; likewise P_{III} is able to cleave bond 13–14. Since the products found are complementary, they seem mainly to be the result of a primary action on α_{s1}-CN(f1–23). In a later stage of the degradation, when α_{s1}-CN(f1–23) has almost completely disappeared, secondary conversion involving initial products takes place in the case of P_I. The fragment f(1–13) is converted, apparently with formation of f(1–8) and f(1–9). Also the concentration of primary *C*-terminal products decreases slowly. A secondary conversion of initial products by P_{III} is less evident.

These results indicate that, under the present conditions, each enzyme shows a clear preference for bonds in the substrate which are hardly, or not, cleaved by the other enzyme. The specificity of P_I towards α_{s1}-CN(f1–23) shows similarity with that already established with β-casein (Visser *et al.*, 1988) and with that found with other P_I-type proteinases (Monnet *et al.*, 1989). The different types of peptide bonds recognized among a total of 23 cleaved by P_I are summarized in Table 2, and the following deductions can be made. The P_1' residue *C*-terminal to the scissile bond seems arbitrary, although a proline residue is not likely to occur. In many cases cleavage concerns a peptide bond with the (relatively

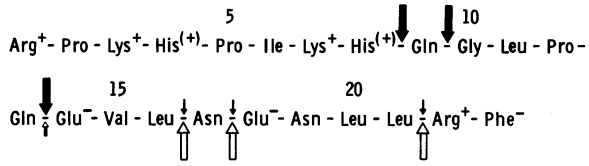

Fig. 2. Amino acid sequence of α_{s1}-CN(f1–23), showing the cleavage sites of the P_I-type (⇓) and of the P_{III}-type (⇑) proteinase

The corresponding small arrows indicate the bonds which are cleaved by P_I and P_{III} at a relatively low rate.

hydrophilic) glutamine residue in the P_1 position (type 2 and 8; ten bonds). In cases where P_1 is not glutamine, it is either a hydrophobic residue (type 1, 3 and 6; eight bonds) with a side-chain volume close to that of glutamine [0.095 nm^3 (95 $Å^3$)] or larger, or it is the relatively small serine residue [volume 0.033 nm^3 (33 $Å^3$)] (type 4 and 5; four bonds). If glutamine is in the P_1' position, the P_1 residue is either very hydrophobic (type 3) or, again, a serine residue (type 5) or, in one case, a positive charge is present *N*-terminally not far from the scissile bond and even histidine appears in the P_1 position (type 7). If serine is in the P_1' position, the P_1 residue is either glutamine or a hydrophobic residue (types 1 and 2).

Although the features of the P_1 residues are very different, they must, nevertheless, be most suitable to fit into, in one way or another, the P_1 binding site. A model of the substrate-binding region, as predicted on the basis of a close sequence similarity with the subtilisin family (Vos *et al.*, 1989; Wells *et al.*, 1987) is presented in Fig. 3. A close examination of the three-dimensional structure of the P_1 binding site shows that it is a large cleft with hydrophobic backbone walls and a bottom represented by a glycine residue (Wells *et al.*, 1987). Nearly all of the residues

Table 2. Representation of the different types of peptide bonds in β-casein (β-CN) (Visser *et al.*, 1988; Monnet *et al.*, 1989), chromophoric peptides (Exterkate, 1990) and in α_{s1}-CN(f1–23) (the present work) cleaved by the P_I-type proteinase

The most essential features of the amino acid sequence around the scissile bond are shown. The side chain of the omitted residues in each of the positions P_2–P_4 is in almost all cases either hydrophobic or able to take part in a hydrogen bridge (that is, serine, threonine or glutamine). Proline is frequently found in one or two of these positions. For further details, see the text.

P_4	—	P_3	—	P_2	—	P_1	(Hφ*/Vol.†)	-----	P_1'‡	Substrate	Type
						Phe	(+2.5/137)	-----	Xaa§(= Ser-┼-)‖	β-CN	1
						(Asn)Gln	(−0.3/95)	-----	Xaa (= Ser-┼-)	β-CN/α_{s1}-CN(f1–23)	2
						Tyr	(+2.3/138)	-----	Gln	β-CN	3
						(Thr)Ser	(−0.3/33)	-----	Xaa	β-CN	4
						Ser	(−0.3/33)	-----	Gln	β-CN	5
Ile	—	$His^{(+)}$	—	Pro	—	Phe	(+2.5/137)	-----	Xaa	β-CN	6
Pro	—	Ile	—	Lys^+	—	$His^{(+)}$	(+0.5/101)	-----	Gln	α_{s1}-CN(f1–23)	7
Ile	—	Lys^+	—	His^+	—	Gln	(−0.3/95)	-----	Xaa	α_{s1}-CN(f1–23)	8
MeOsuc¶—		Arg^+	—	Pro	—	Tyr	(+2.3/138)	-----	pNA**		

* Hydrophobicity of the amino acid side chain in kcal (note 1 kcal = 4.18 kJ)/mol (Chothia, 1984).
† Mean volume of amino acid side chain buried in proteins [in $Å^3$ (1Å = 0.1 nm)] obtained by subtracting the volume of a glycine residue (Chothia, 1984).
‡ Peptide residue nomenclature according to Schechter & Berger (1967).
‖ Also cleaved by P_I-type proteinase (that is, type 4).
§ Can be various residues, but not proline.
¶ Methoxysuccinyl.
** *p*-Nitroanilide.

forming this cleft are conserved in subtilisin and lactococcal proteinases. This cleft can accommodate relatively large hydrophobic residues (Estell *et al.*, 1986) and, indeed, phenylalanine and tyrosine are found as P_1 residues (Table 2). The presence of serine-193 at the end of the cleft and the amide group in the P_1 glutamine side chain of the substrate might be most favourable for the formation of (a) hydrogen bridge(s) in addition to a (weak) hydrophobic backbone interaction. On the other hand, small P_1 residues that carry an H-donor group (that is, serine, threonine and possibly also cysteine) may be found effectively by the formation of a hydrogen bond involving an acceptor group at the entrance of the cleft, possibly asparagine-196. A favourable orientation might be stabilized in that way.

Taken together, these results suggest that the specificity of the enzyme with regard to the P_1 substrate residue is not very exclusive. However, optimal binding apparently occurs in the case of a strongly hydrophobic, a glutamine or a serine residue. In the case where P_1 binding is less effective, the features of more distantly positioned substrate residues (P_2–P_4) might be more important in determining the efficiency of binding.

The latter residues (that is, P_2–P_4) are in general hydrophobic; frequently proline occurs in one or two of these positions. If these residues are not hydrophobic, the side chain is in most cases able to take part in a hydrogen bridge (that is, serine, threonine or glutamine), or a positive charge is also present at position P_2 and/or P_3.

The nature of these side chains reflects the properties of residues in the enzyme molecule which are considered important for binding, that is, residues 136–144 and 162–166, corresponding to residues 99–107 and 125–129 of subtilisin respectively (Robertus *et al.*, 1972; Wells *et al.*, 1987) (Fig. 3).

Specific cation-binding involving aspartic acid-166, which seems to be important for small-peptide binding (Exterkate, 1990), appears not to be a determinant in the cleavage of β-casein and α_{s1}-CN(f1–23) by proteinase P_I. However, it is striking that the cleavage sites are in the positively charged *N*-terminal part of α_{s1}-CN(f1–23) and in hydrophobic parts of β-casein. In none of the cases of preferred cleavage sites in both substrates is a negative charge present at the *N*-terminal side close enough to

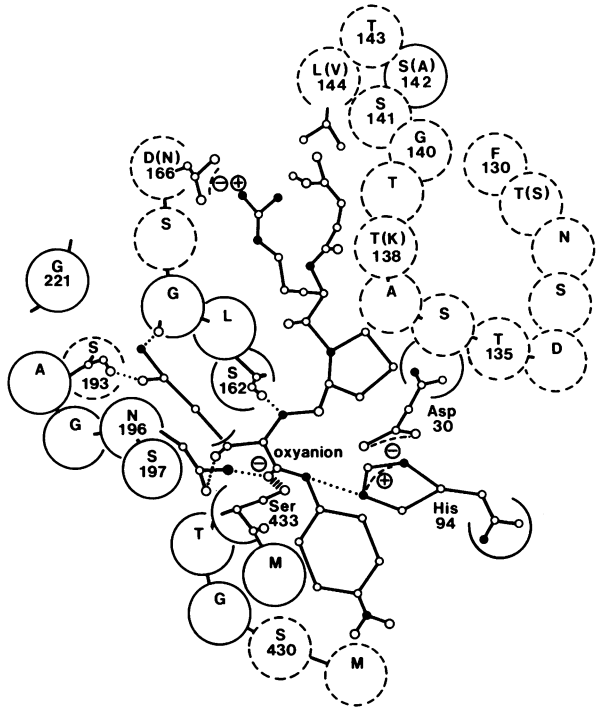

Fig. 3. Model of the binding region of proteinase P_I (showing also, in parentheses, the substitutions in P_{III}) predicted on the basis of a close sequence similarity with the subtilisins (Vos *et al.*, 1989)

Residues of the catalytic triad (Ser^{433}, His^{94} and Asp^{30}) and other pertinent residues involved in binding are represented in more detail. The interaction with a model substrate methoxysuccinyl-Arg-Pro-Gln (or Ser) *p*-nitroanilide and the possible formation of hydrogen bonds (dotted lines) involving the glutamine or serine residue in the P_1 position are shown. All residues presented as closed circles are conserved in subtilisin BPN′ and lactococcal proteinases. The model is based on that presented by Wells *et al.* (1987). Key to symbols: ○, $-CH_2-$ or $-CH_3$; ●, $-NH-$, $-NH_2$ or $=NH$; ○, $=O$ or $-OH$.

interfere with binding. Apparently a general cation attraction and/or hydrophobic interaction and hydrogen-bond formation are important in the first place.

In those cases of a relatively low cleavage rate, an *N*-terminal negatively charged residue is too close (that is, in position P_3 or P_4), and electrostatic repulsion may occur. This view is sustained by the effects of salt and pH on specificity (F. A. Exterkate & A. C. Alting, unpublished work). Interestingly, these poorly cleaved peptide bonds in the case of P_I action on α_{s1}-CN(f1–23) appear to be the preferred bonds for the action of P_{III}. Since the P_1 binding cleft in P_{III} is identical with that of P_I, the same features of the substrate residue at position P_1 are important. Apparently also in this case an electrostatic repulsion/attraction can play a role in selecting the peptide bonds to be cleaved, and that is why only bond 13–14 is also split by P_{III}, although at a much lower rate than the preferred bonds.

The above considerations with respect to differences between P_I and P_{III} specificity are in line with characteristic features of the respective binding regions outside the P_1 binding site. These regions differ with respect to the charge of residues which, on the basis of the close sequence similarity of these proteinases with subtilisin and their predicted three-dimensional structure (R. J. Siezen, P. A. J. Vos, W. M. de Vos & B. W. Dijkstra, unpublished work), are supposed to be involved in the (backbone) interaction with the substrate residue P_3 (Robertus *et al.*, 1972; Wells *et al.*, 1987), namely aspartic acid-166 and threonine-138 in P_I have been replaced by asparagine and lysine respectively in P_{III}. In the model the side chains of these residues both point upwards and can interact with the P_3 side chain, which points in the same direction. The P_4 side chain is in a downward position. It appears from this that, under the conditions present in cheese (e.g. at relatively high salt concentration and a low pH), significant effects on specificity might be expected. The present results can be used to study these effects and to learn more about the fate of α_{s1}-CN(f1–23) and its hydrolysis products during the ripening of cheese.

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