

## Milk peptides: effect on the enzymatic hydrolysis of sodium caseinate

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Sodium caseinate (NaCN) was hydrolyzed using Rhozyme 41 (Rh<sub>41</sub>), Neutrase (Neu) and plasmin (PL) to obtain peptide preparations termed; Na-Cas-P-Rh<sub>41</sub>, Na-Cas-P-Neu, and Na-Cas-P-Plasmin, respectively. Indigenous whey peptides (IWP) were obtained from fresh sweet whey, at different levels of uf-concentration, by a precipitation method described earlier. These peptide fractions were then used to observe their effect on the activity of some proteases. All peptide preparations depressed enzyme activity. Na-Cas-P-Rh<sub>41</sub> was the most powerful inhibitor of enzyme activity and decreased the activity of trypsin, Rh<sub>41</sub>, Neu, and PL by 15, 32, 50, and 14%, respectively. IWP markedly depressed activity of Rh<sub>41</sub>. The degree of uf-concentration of whey from which IWP was obtained was directly related to degree of inhibition.

Key words: peptide, casein, cheese, ripening, enzyme

### Introduction

Enzymes play key roles in the manufacture of many foods (BEST 1987, WONG et al. 1988). Dairy foods like yogurt, cheese, buttermilk, sour cream, acidophilus milk, Kefir cultured milk, and Kumiss are partly or fully dependent on enzyme action for the formation of desirable flavor and physico-chemical attributes (BEST 1987). The action of enzymes on dairy proteins (VISSER 1981), and the classification and mode of action of proteases in general, have been extensively reviewed (MORIHARA 1974, VISSER 1981, NEURATH 1985, YELLIN 1986).

Proteolytic activity is particularly important in the manufacture and aging of cheese (LAW and WIGMORE 1982, PAHKALA et al. 1984). In order to accelerate the ripening process of cheese, various enzyme preparations are added (EXTERKATE et al. 1987, FOX 1989) at different stages of the process

(LAW and WIGMORE 1982, LINDE et al. 1989). Caseins form the network matrix of the cheese and contributes to the flavor. The rate and extent of hydrolysis of caseins influence the quality of cheese (ZEVACO and DESMAZEAUD 1980, BANKS et al. 1988, CHRISTENSEN et al. 1989). Enzymes have thus been used to influence both the quality and aging time of cheeses (LAW 1981, LAW and WIGMORE 1983). Unfortunately, use of enzymes may lead to the formation of off-flavors and of bitter peptides (FOX 1989). Bitter peptides detected from cheese are small and hydrophobic (VISSER et al. 1983, HURRELL et al. 1989). Such protein fragment are more likely to occur if the enzyme activity is high and the action is thorough.

Since the formation of the enzyme-substrate complex, i.e., protein-protein association, is a prerequisite for enzyme activity, molecules that interfere with the complex formation decrease/inhibit

activity. Amphipathic peptides produced following enzyme hydrolysis of casein reduced protein-protein association (HAQUE and BOHOUA 1990). It would be useful for the industry, particularly for the cheese makers, to know whether such peptides may influence activity of ripening enzymes.

The objective of this part of the study was therefore to evaluate the influence of casein hydrolysates on the activity of some food enzymes. We have used uptake of base (NaOH) as an indicator of the rate of hydrolysis. A pH-stat was used to monitor uptake as described earlier (ANTILA et al. 1987). The theoretical basis for the pH drop method in non-buffered suspensions has been elaborated (HUNG et al. 1984). Trypsin was chosen since it is considered safe in food applications and have been extensively studied, plasmin was used since it too has been well studied and is known to effect cheese quality (LE-BARS and GRIPON 1989), and Neutrased and Rhozyme-41 were selected randomly from cheese ripening enzymes.

## Material and methods

Fresh milk was obtained from the MSU Dairy Plant. Bio-Rad protein assay dye kit was obtained from Bio-Rad Chem. Div. Richmond, CA. Recrystallized urea, imidazole, 2-mercaptoethanol (2-ME), plasmin (P-7911)(EC 3.4.21.7) (PL), pepsin (P-6887) (EC 3.4.23.1) (PEP), and trypsin (T-8253) (EC 3.4.21.4) (TRY) were obtained from Sigma Chem. Co., St. Louis, MO. Rhozyme-41 (Rh-41) concentrate (factor 6.15) a proteolytic enzyme mixture was obtained from Genencor, South San Francisco, Ca. Neutrased (NEU), a proteolytic enzyme mixture (batch #PW20502, activity 1.63 unit/g) was obtained from Novo Industries, Copenhagen, Denmark. Standard base for pH-stat was from E. Merck, Darmstadt, Germany. All other chemicals were analytic grade and obtained from recognized sources.

## Preparation of sodium-caseinate (NaCN)

Fresh raw milk (1 l) was centrifuged at about 3 000 x g to remove fat and diluted 2 fold with water. The

pH was then adjusted to 4.6 with 1 N HCl at 25°C and the resulting coagulum was filtered with a cheese cloth after standing for 30 min. The casein thus obtained was washed and then redispersed in distilled water (1 l) at pH 4.6 and again filtered after 60 min. The process of washing and redispersion was repeated. The acid casein was redispersed in distilled water by adding 1 M NaOH to adjust pH to 7, acid precipitated followed by washing with acidified water. This final coagulum was redispersed in water at pH 7 (25°C) (adjusted with NaOH) and freeze dried after flash freezing in liquid nitrogen. The NaCN powder thus obtained was stored over desiccant at -20°C until required.

## Preparation of milk peptides

### *Milk protein hydrolysate*

Casein hydrolysate were prepared from NaCN by enzyme action using PL, Rh-41, Neu, TRY, and PEP under conditions described in Table 1. NaCN (final concentration 3%, w/v) was hydrated for an hour in distilled water, the pH was adjusted using 0.1N HCl or NaOH, temperature was equilibrated to 37°C, and the freshly prepared aqueous enzyme solution was added to bring the final enzyme:protein concentration to 1:200. The hydrolysis was carried out for periods as listed in Table I and the reaction was stopped by heating at 85°C for 15 min. The hydrolysate was flash frozen using liquid nitrogen, freeze dried and stored over desiccants at -20°C until needed. The peptides thus obtained were termed as follows: Na-Cas-P-Rh<sub>41</sub>, Na-Cas-P-Neu, and Na-Cas-P-Plasmin for peptides generated from Rh<sub>41</sub>, Neu and PL, respectively.

### *Indigenous milk peptides*

Indigenous peptides in sweet whey (Edam) was concentrated by ultrafiltration (Romicon, 10 k-cut) to different concentration (1, 1.5 and 4 fold) and dried by freeze drying to obtain free flowing powders. The peptides in the WPC were isolated by a modification of the method of HAQUE and KINSELLA (1988) (Figure 1) as follows: The WPC pow-

Table 1. Preparation of crude peptides from milk proteins. Reactions were stopped by heating to 85°C for 15 min.

Enzymes	Enz:protein ratio	Time	pH
plasmin	1:200	3	7.5
rhozyme 41	1:200	3	7
neutrase	1:200	3	6

der (10 g) was extracted twice (150 ml x 2) with acidified methanol containing 0.1 % (v/v) redistilled HCL (single boiling point). Solids in the extract were removed by centrifugation (1000 g) and the supernatant was dried in partial vacuum at 35°C to yield an oily residue that was washed thrice (20 ml x 3) with cold diethyl ether (-20°C) followed by further washing (3 x 20 ml) with cold acetone:diethyl ether (1:1, v/v). The resulting peptide mixture was dried in a stream of nitrogen and stored in vacuum over phosphorus pentoxide until needed.

### Monitoring the digestibility of casein

Proteolysis of sodium caseinate (NaCN) was monitored using a pH-stat (Mettler, Memotitrator DL 40RC) attached to a chart recorder and a Mettler GA 44 printer. Optimum temperature was maintained by using a jacketed reaction vessel connected to a controlled temperature water bath. The full scale of the pH-Stat was equal to 400 µl of 0.05 M NaOH unless otherwise stated in the text.

The NaCN (3%, w/v) (20 ml) was allowed to equilibrate to optimum temperature (37°C). Freshly prepared enzyme solution in cold water was then added (0.3 ml) with constant stirring to bring the protein to enzyme ratio to 1:200, w/w. The uptake of base over a period of 300 sec was recorded.

### Effect of peptide on proteolysis

The effect of the various peptide preparations on the digestibility of NaCN was observed after adding 10 and 20% by weight of the peptide preparations to

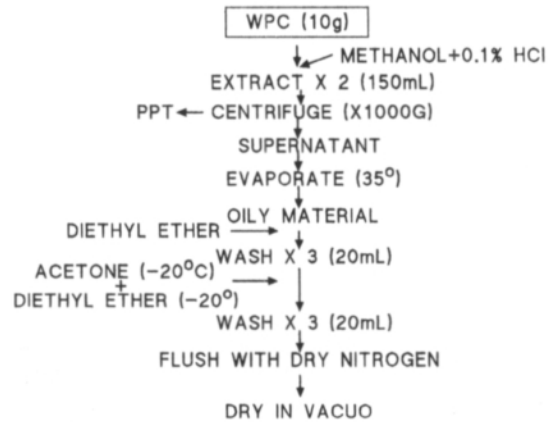


Fig. 1. Scheme of the isolation of indigenous milk peptides from whey protein concentrates (WPC) of various concentrations.

the protein dispersions. The mixture was mixed uniformly by "vortex" mixing and sonication prior to the experiment.

### Monitoring of hydrolysis

#### Polyacrylamide gel electrophoresis

Very high-resolution polyacrylamide gel electrophoresis (VHR-PAGE) was carried out in the presence of SDS using a 10-20% gradient gel (HAQUE and MOZAFFAR 1992a, b).

#### High performance liquid chromatography (HPLC)

HPLC of the hydrolysates was performed with a Bondacelone 10 C<sub>18</sub> column (60 cm x 0.7 cm) (Phenomenex, CA) as described elsewhere (HAQUE and MOZAFFAR 1992b). Samples were eluted with a linear gradient where buffer A was 0.11 wt% trifluoroacetic acid in double distilled water, and B was 90 wt% acetonitrile in water containing 0.1 wt% trifluoroacetic acid. An ISCO ChemResearch System containing a model 2360 gradient programmer (ISCO, Inc. USA) coupled with pressure regulators (Model 2350), V<sup>4</sup> absorbance detector (ISCO), and refrigerated ISIS autoinjector, was used for monitoring the hydrolysis.

*Determination of the N-Terminal Groups*

The degree of hydrolysis as a function of the free N-terminal groups was determined by the modified OPA method of FRIESTER et al. (1989) (Table 2).

**Results and discussion**

Polyacrylamide gel electrophoresis showed highest apparent degree of hydrolysis of casein by Rh<sub>41</sub> (Figure 2). The PAGE of hydrolysis with TRY, a powerful protease, has been shown as a comparison. It is clear that Rh<sub>41</sub> was apparently as powerful, if not more powerful, than TRY. Hydrolysis by PL and NEU was much slower (data not shown). HPLC confirmed this observation for Rh<sub>41</sub> (Figure 3) whereas peptides for NEU and PL were in trace amounts (data not shown). Based on the availability of N-terminal groups, the degree of hydrolysis was Na-Cas-P-Rh<sub>41</sub> > Na-Cas-P-Neu > Na-Cas-p-plasmin (Table 2).

Na-Cas-P-Rh<sub>41</sub> and Na-Cas-P-Neu markedly reduced the rate of digestion of caseinate by TRY (Fig. 4). When the peptide concentration was increased to 20% (w/w casein), there was a concomitant increase in the inhibitive effect; Na-Cas-P-Rh<sub>41</sub> decreased activity rate of TRY by about 15% at an incubation time of 300 sec. The order of the depressing effect of the peptides remained the same; Na-Cas-P-Rh<sub>41</sub> > Na-Cas-P-NEU > Na-C-P-Plasmin. This is a significant observation because trypsin is a powerful protease (Fig. 2), and we have used an enzyme to protein concentration of 1:200 which is relatively high.

When Rh<sub>41</sub> was used to hydrolyze casein, the most active activity suppressants were again Na-Cas-P-Rh<sub>41</sub> and Na-Cas-P-Neu, the latter being

Table 2. Degree of proteolysis of crude peptide preparations from sodium caseinate. Determined by the method of FRIESTER et al. (1989).

Peptide	Enzyme	Degree, %
Cas-P-Plasmin	plasmin	<1
Cas-P-Neut	netrase	7.0
Cas-P-RH41	rhozyme-41	9.2

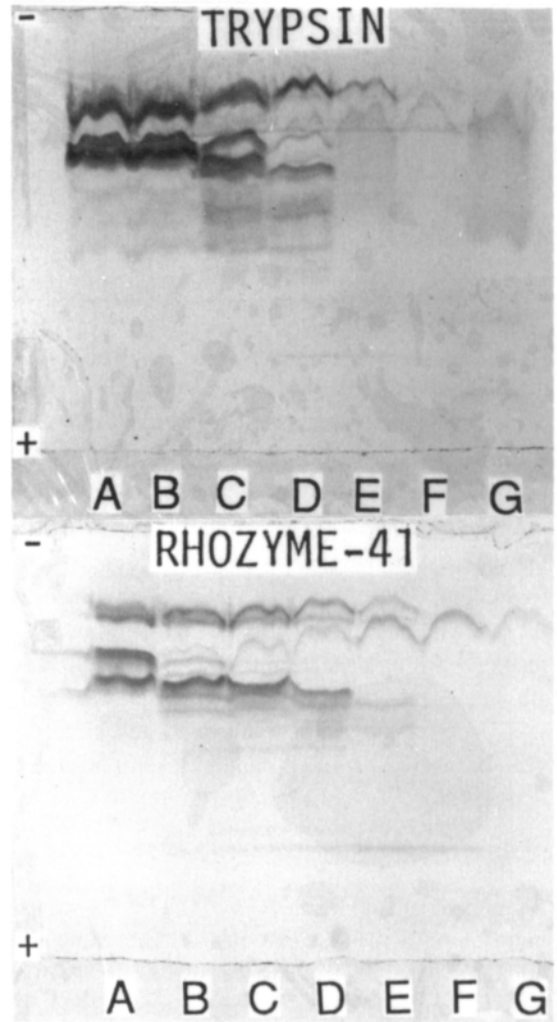


Fig. 2. Polyacrylamide gel electrophoresis of casein hydrolysates. A, B, C, D, E, F and G represent control, 10, 20, 30, 60, 120, 180 and 240 min samples, respectively. (Photo: Z. U. Haque.)

more potent (Fig. 5). This effect was markedly enhanced when the peptide content was increased to 20% (w/w casein); Na-Cas-P-Rh<sub>41</sub> decreased the activity rate by almost 32%. Data indicate that the hydrolytic activity of this enzyme is very much inhibited by the peptides this enzyme itself generates. Earlier, we observed that Na-Cas-P-Rh<sub>41</sub> are highly amphipathic and surface active (soap-like) (HAQUE 1991a, b). On the contrary, Na-Cas-P-Plasmin appeared to elevate the activity slightly at all stages of the reaction. This was true at both the

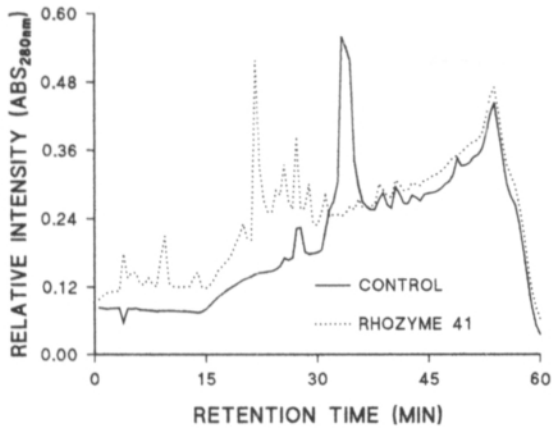


Fig. 3. Reverse phase high performance liquid chromatography of casein hydrolysate using Rh<sub>41</sub>. Peptide peaks with low retention time are smaller and/or less hydrophobic.

peptide concentrations. We have previously observed that PL produces comparatively large and highly surface active peptides from casein (HAQUE 1991). Perhaps these peptides were a ready substrate for Rh<sub>41</sub>.

The precursor for PL is indigenous to milk. Thus it is possible that PL indirectly influences flavor and texture by providing large peptides that easily broken down by other proteases in milk. It has been reported that plasmin digestion of casein markedly influences the quality of cheese and other dairy products (BASTIAN *et al.* 1991).

To further understand the influence of slow acting enzymes that are typically used in the accelerated cheese ripening processes, Neu, a neutral peptidase used in accelerated cheese ripening, was used next. The uptake of sodium hydroxide after 300 seconds of incubation time was only 14 mole per gram of protein showing that this enzyme was slow compared to Rh<sub>41</sub> and TRY. The effect of the peptides on activity of NEU was the most marked compared to all the other enzymes tested. At the lower concentration of 10%, the depression in activity was in the order Na-Cas-P-Neu > Na-Cas-P-Rh<sub>41</sub> > Na-Cas-P-Plasmin. At the higher concentration of peptides (20%), Na-Cas-P-Rh<sub>41</sub> was the most detrimental and depressed Neu activity rate by 50% at an incubation time of 300 sec (Fig. 6).

The activity of PL is very low to begin with. Added peptides further depressed the activity by a maximum (20% peptide, w/w protein) of about 14% at an incubation time of 300 sec. At the higher peptide concentration, the depression of activity was clearly seen, for Na-Cas-P-Rh<sub>41</sub> and Na-Cas-P-Neu, throughout the incubation (Fig. 7).

Data indicate that in all the above enzymes, Na-Cas-P-Rh<sub>41</sub> was the most effective in depressing activity. The inhibitive activity on a percentile basis was the cheese ripening enzymes (NEU and Rh<sub>41</sub>).

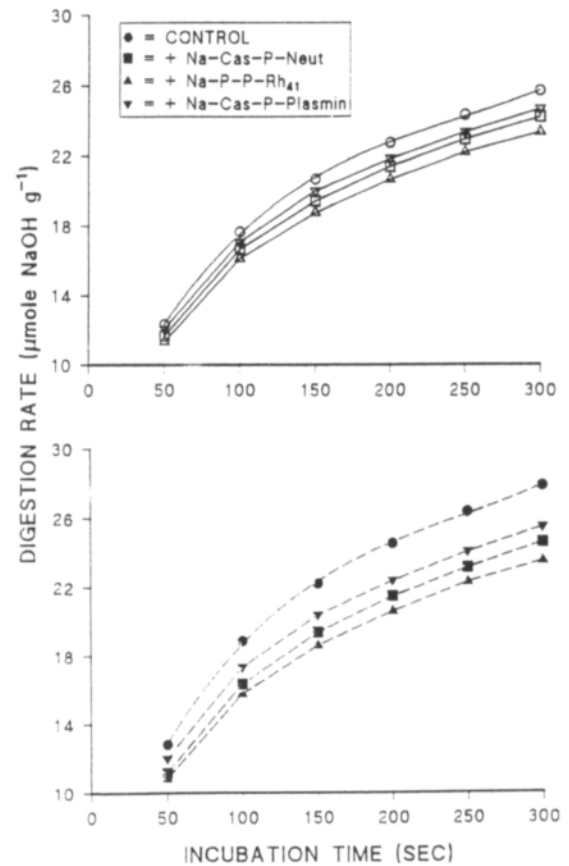


Fig. 4. Effect of various milk peptides on the digestibility of sodium caseinate with trypsin. The peptide concentration was 10% (open symbols) and 20% (solid symbols) (wt/wt of protein). The control samples had an equal amount of added sodium caseinate that had been passed through all the steps used in the production of peptides without the addition of the enzyme. Freshly prepared enzyme solutions were used at a enzyme to protein ratio of 1:200. The X-axis represents the incubation time at 37°C and the Y-axis is the digestion rate.

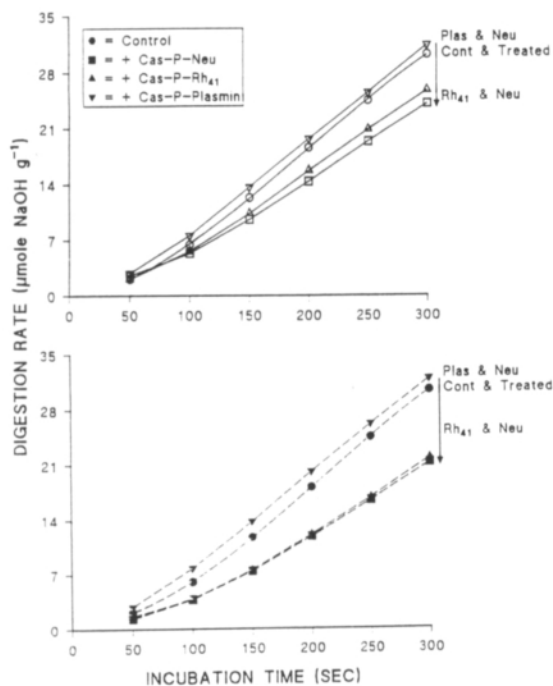


Fig. 5. Effect of various milk peptides on the digestibility of sodium caseinate with plasmin. All conditions and descriptions are the same as in Fig. 4.

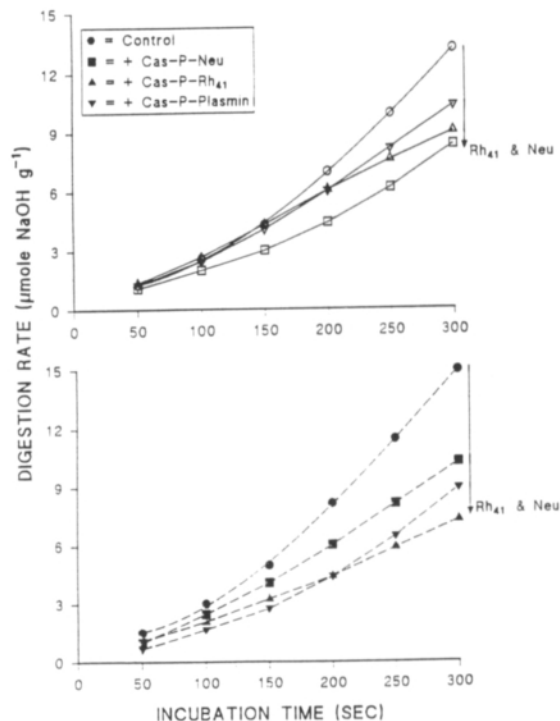


Fig. 6. Effect of various milk peptides on the digestibility of sodium caseinate with Rhozyme 41 (Rh<sub>41</sub>). All conditions and descriptions are the same as in Fig. 4.

**Effect of indigenous milk peptides on the digestibility of caseinate**

The crude peptide preparations from WPC markedly reduced the activity of Rh<sub>41</sub> (Fig. 8). This depression of enzyme activity was directly related to the concentration (ultrafiltration {UF}) of the whey proteins at a peptide concentration of 10% (w/w protein). Data indicate that certain peptides that are concentrated by UF concentration inhibit the activity of Rh<sub>41</sub>. When powerful protease, TRY, was used, the activity was modulated by the UF

peptides but they did not seem to be directly affected.

The amphipathic nature of whey peptides has been illustrated by us earlier (HAQUE 1991, 1993). More recently, we have isolated and sequenced the major amphipathic peptides from whey. The major peptide originates from β-casein (HAQUE et al. 1993).

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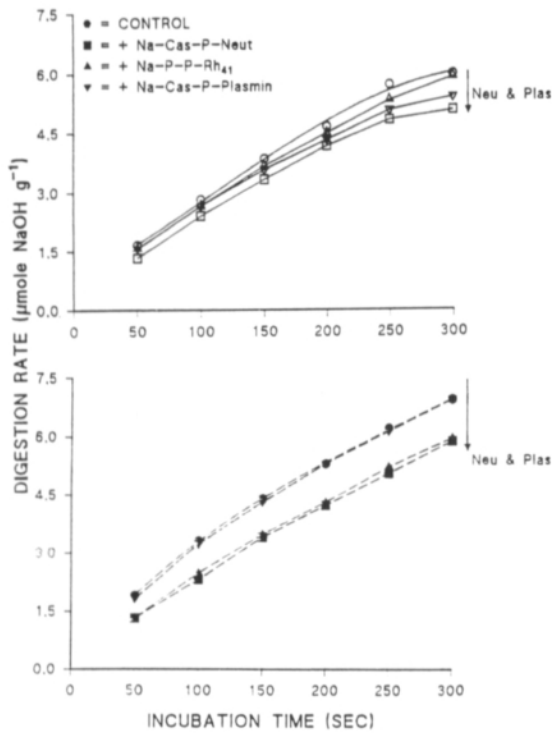


Fig. 7. Effect of various milk peptides on the digestibility of sodium caseinate with Neutrase. All conditions and descriptions are the same as in Fig. 4.

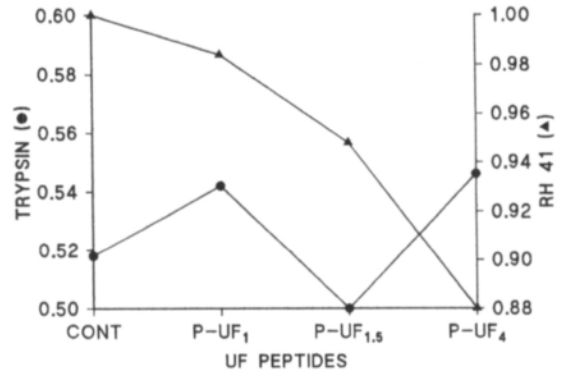


Fig. 8. Effect of indigenous peptides (P) on the digestibility of sodium caseinate. The peptides were obtained as described in Fig. 1 from whey protein concentrate (WPC), at various levels of concentration. The enzymes used were trypsin and Rhozyme 41 (Rh<sub>41</sub>), a cheese ripening enzyme. The labels on the Y-axis describe the peptides obtained from WPC at various levels of concentration by ultrafiltration (UF), i.e., 1 fold is UF<sub>1</sub>, 1.5 fold is UF<sub>1.5</sub>, and 4 fold is UF<sub>4</sub>. The Y-1 and 2-axis represent the hydrolysis rate for trypsin and Rh<sub>41</sub>, respectively.

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## SELOSTUS

### Maidon valkuaisen peptidit: vaikutus natriumkaseinaattiin

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Tutkimuksessa hydrolysoitiin natriumkaseinaatti (NaCN) Rhozyme41:llä (Rh<sub>41</sub>), Neutrasilla (Neu) ja plasmiinilla (PL), jolloin saatiin seuraavat peptidiyhdisteet: Na-Cas-P-Rh<sub>41</sub>, Na-Cas-P-Neu ja Na-Cas-P-PL.

Alkuperäiset herapeptidit (indigenous whey proteins = IWP) saatiin tuoreesta herasta saostusmenetelmällä. Näitä peptidifraktioita käytettiin tutkittaessa maidon peptidien

vaikutusta eräiden proteaasien aktiivisuuksiin. Kaikki tutkitut peptidifraktiot alensivat entsyymiaktiivisuuksia. Na-Cas-P-Rh<sub>41</sub> oli voimakkain entsyymiaktiivisuuden heikentäjä, sillä se alensi trypsiini-, Rh<sub>41</sub>-, Neu- ja PL-aktiivisuuksia 15, 32, 50 ja 14 %. IWP alensi selvästi Rh<sub>41</sub>-aktiivisuutta. Heran ultrasuodatus-konsentraatitaso oli suoraan verrannollinen entsyymi-inhibition voimakkuuteen.