Z Ernährungswiss 34: 118–130 (1995)


ORIGINALARBEIT

P. Chr. Lorenzen
E. Schlimme

# Characterization of trypsin immobilized on oxirane-acrylic beads for obtaining phosphopeptides from casein

**Charakterisierung von an Oxiran-Acrylharzperlen gebundenem Trypsin zur Gewinnung von Phosphopeptiden aus Casein**

**Summary** The aim of the study was to characterize the proteolytic properties of immobilized trypsin for obtaining phosphopeptide-rich fractions from casein. Trypsin was covalently bound to oxirane-acrylic beads. After incubation for 48 h immobilization degrees of about 85 % were achieved. 20 % of the immobilized enzyme exhibited no activity towards the substrate N-benzoyl-L-arginine ethyl ester.

Compared with homogeneous catalysis with the soluble enzyme a 25 % lower degree of proteolysis was calculated and a modified peptide pattern of the resulting proteolysates established. A caseinophosphopeptide (CPP) from $\alpha_{s1}$-CN (59–79) was not detectable after incubation with the carrier-bound enzyme.

At a substrate concentration (S) of 15 % (w/w) substrate saturation of the enzyme (E) was achieved. Increasing the substrate concentration to 20 % (w/w) decreased the conversion rates (content of soluble amino-N) and the liberation of CPPs. Proteolysis of small (1 % w/w) and partly also large (20 % w/w) substrate concentrations (E/S = 1/100) is subject to changed kinetic conditions. The same was true for small and large enzyme concentrations (S = 5 % w/w). Compared with enzyme saturation (E/S = 1/50), lack of enzyme (E/S = 1/800) led to a disproportional decrease in the proteolysis rate and to a markedly decreased content of hydrophobic peptides in the resulting proteolysates. Increasing the pH from 7.8 to 8.8 and the temperature from 37 ° to 47 °C caused only a slight increase in conversion rates, but an overproportional liberation of CPPs (pH 8.8 = + 47 %, 47 °C = + 89 %), in particular from β-casein. Repeated use of immobilized trypsin resulted after nine runs in a loss in proteolytic activity and in CPP yields of approximately 25 %, while the peptide pattern of the proteolysates remained qualitatively unchanged. Light microscopy shows that the oxirane-acrylic beads disintegrate to a large extent after nine repetitions.

Received: 28 November 1994
Accepted: 18 January 1995

Dr. P. Chr. Lorenzen (✉) · E. Schlimme
Bundesanstalt für Milchforschung
Institut für Chemie und Physik
Postfach 60 69
24121 Kiel

**Zusammenfassung** Gegenstand der Arbeit war die Charakterisierung der proteolytischen Eigenschaften von immobilisiertem Trypsin in der Gewinnung phosphopeptidreicher Fraktionen aus Casein. Trypsin wurde kovalent an Oxiran-Acrylharzperlen gebunden. Nach 48stündiger Inkubation wurden Immobilisierungsgrade um 85 % erreicht. 20 % des immobilisierten Enzyms wies gegenüber dem Substrat N-Benzoyl-L-Arginin-Ethyl-Ester keine Aktivität auf.

Im Vergleich zur homogenen Katalyse (gelöstes Enzym) wurde ein um 25 % geringerer Proteolysegrad errechnet und ein verändertes Peptidmuster der resultierenden Proteolysate festgestellt. Ein Caseinophosphopeptid (CPP) aus $\alpha_{s1}$-CN (59–79) war nach Inkubation mit dem trägergebundenen Enzym nicht nachweisbar.

Bei einer Substratkonzentration von 15 % (w/w) wurde Substratsättigung des Enzyms erreicht. Eine Erhöhung auf 20 % (w/w) führte zu einem Abfall der Umsatzraten (Gehalt an löslichem Amino-N) und einer verringerten Freisetzung an CPPs. Die Proteolyse bei geringen (1 % w/w) und punktuell auch bei hohen (20 % w/w) Substratkonzentrationen (E/S = 1/100) unterliegt veränderten kinetischen Bedingungen. Der gleiche Sachverhalt wurde bei geringen bzw. hohen

Enzymkonzentrationen (S = 5 % w/w) festgestellt. Enzymmangel (E/S = 1/800) führte – im Vergleich zur Enzymsättigung (E/S = 1/50) – zu einer unproportionalen Abnahme der Proteolyserate und zu einem deutlich verringerten Gehalt an hydrophoben Peptiden in den resultierenden Proteolysaten. Die Erhöhung des pH-Wertes von 7,8 auf 8,8 bzw. der Temperatur von 37 °C auf 47 °C führte zwar nur zu einer geringen Steigerung der Umsatzraten, aber zu einer überproportionalen Freisetzung an CPPs (pH 8,8 = + 47 %, 47 °C = + 89 %), insbesondere aus β-Casein. Die Mehrfachnutzung des immobilisierten Trypsins ergab nach neunfacher Anwendung einen Verlust der proteolytischen Aktivität und der Ausbeute an CPPs von ca. 25 %, das Peptidmuster der Proteolysate blieb aber qualitativ unverändert. Lichtmikroskopische Aufnahmen haben gezeigt, daß die Oxiran-Acrylharzperlen nach neunfacher Anwendung weitestgehend zerbrochen waren.

**Key words** Immobilization – trypsin – caseinophosphopeptide – variation of process parameters

**Schlüsselwörter** Immobilisierung – Trypsin – Caseinophosphopeptide – Variation von Prozeßparametern

**Abbreviations** $\alpha_{s1}$-*CN* = alpha$_{s1}$-casein · β-*CN* = beta-casein · *BAEE* = N-benzoyl-L-arginine ethyl ester · *CPP* = caseinophosphopeptide · *E* = enzyme · *E/S* = enzyme-substrate ratio · *HPLC* = high performance liquid chromatography · *IE* = ion exchange chromatography · *S* = *substrate concentration*

## Introduction

Casein tryptic peptides containing multiple phosphoseryl residues have potential as additives for the prevention of dental diseases in toothpaste, mouthwash, and food (1). Such products, however, are not yet commercially available. Normally, soluble proteinases are used to liberate CPPs enzymatically from casein (7, 22). Although there has been increased interest in the use of immobilized enzymes because of their greater stability to both temperature and pH, as well as the possibility of recovering and reusing the enzyme (8), their application for the preparation of CPPs has not yet been described. Our previous studies on in vitro proteolysis of casein with soluble trypsin or trypsin immobilized on oxirane beads (11) have shown, however, that the use of immobilized enzyme leads under comparable conditions to significantly less hydrolysis and to greater chain lengths of the peptide fragments. Furthermore, separation of the proteolysis products with an ion exchanger has shown that the soluble enzyme generates a CPP that is not eluted if the immobilized enzyme is used.

The purpose of this study was to characterize possible changes in the peptide pattern attributable to the immobilization of the enzyme, and to determine the extent to which the proteolytic properties and therefore the ability to liberate CPPs are influenced by the variation of parameters like substrate concentration, enzyme-substrate ratio, pH, temperature and multiple reuse of the immobilized enzyme.

## Material and methods

### Immobilization procedure

Oxirane beads (Eupergit C, Röhm Pharma GmbH, Weiterstadt, FRG, epoxide content approximately 600 µmol/g dry beads) were used as support material for immobilizing trypsin. Nine g of dry oxirane beads were washed on a D4-glass frit with 1 l of demineralized water and approximately 300 ml buffer (1 mol/l potassium phosphate, pH 7.5); 180 mg trypsin were dissolved in 90 ml buffer and dialyzed against buffer at 4–6 °C for 14–16 h. The solution of trypsin in buffer was quantitatively transferred to the washed oxirane beads and allowed to react at room temperature for 48 h. The immobilization process was monitored photometrically (λ = 280 nm) by measuring the non-bound trypsin in the buffer. The beads with the immobilized enzyme were added to buffer (0.1 mol/l potassium phosphate, pH 7.5, with 500 ppm p-hydroxybenzoic acid ethyl ester and 2 vol% isopropanol) and stored at 4–6 °C. The binding efficiency was calculated from the difference between enzyme addition (180 mg) and the content of non-bound trypsin in the buffer after 48 h (28.3 mg). The difference of 151.7 mg trypsin was bound to 31.5 g of wet oxirane beads (swelling capacity: 2.5 ml of water per gram dry beads). To attain, for example, an enzyme-substrate ratio of 1:100 (w/w), 5.2 g wet beads (= 25 mg trypsin) were added to 2.5 g casein dissolved in demineralized water.

### Proteolysis

Sodium caseinate (Alanate 180, Protein Division New Zealand Dairy Board) was used as substrate for proteolysis. The enzyme preparation used was trypsin from bovine pancreas, TPCK-treated, activity (N-benzoyl-L-arginine ethyl ester, pH 8.0, 25 °C), 40 U/mg, EC, 3.4.21.4 from Merck, Darmstadt, FRG.

Proteolysis of the sodium caseinate solutions was carried out with the pH-stat method (12):

| Parameter | Standard | Variations |
|---|---|---|
| Protein content (%, w/w) in demineralized water | 5 | 1 – 20 |
| Temperature (°C) | 37 | 27 – 52 |
| pH (1 mol/l NaOH) | 7.8 | 6.8 – 9.3 |
| Enzyme-substrate ratio | 1/100 | 1/25 – 1/800 |
| Proteolysis time (min) | 240 | 5 – 240 |

After sedimentation of the beads containing immobilized trypsin the supernatant was decanted, adjusted to pH 4.6 with 1 mol/l HCl and the soluble proteolysis products were separated from the precipitate by filtration (filter: 595 1/2) (11).

Analysis

The peptide patterns of the filtrates obtained by isoelectric precipitation (pH 4.6) were determined by ion exchange chromatography according to the method described by Meisel, Behrens, and Schlimme (14).

Column : heigt 18 cm, diameter 0.9 cm
Resin : Dowex ion exchange resin, p.A., type 1x2, grain size 200–400 mesh counterion $Cl^-$ (Serva, Heidelberg)
Eluents : (1) – 50 ml 0.01 mol/l HCl
(2) – gradient: 200 ml 0.01 mol/l HCl 200 ml 0.1 mol/l HCl
(3) – 100 ml 1 mol/l HCl

Absorption measurements at $\lambda = 210$ nm were carried out with a Shimadzu UV-120-02 photometer, Shimadzu, Düsseldorf. The modified OPA-method (5) was used to characterize the proteolysis products. The method for separating the peptides by reverse phase liquid chromatography has been previously described by Meisel and Frister (15). The comparative studies on the determination of the enzymatic activities of soluble and immobilized trypsin were carried out as described by Merck (18). All results are based on duplicate determinations of two series of independent experiments.

## Results and discussion

Immobilization of trypsin on oxirane-acrylic beads

To follow the immobilization process samples were taken from the immobilization mixture after different periods of time, the oxirane beads removed by membrane filtration and absorption at 280 nm of non-bound trypsin in the filtrate was determined.

The plot in Fig. 1 shows that immobilization is complete after 1 h. The slight increase in absorption at 280 nm after contact times of 24 and 48 h might be explained by the liberation of trypsin molecules that were initially retained on the carrier by non-covalent bonds. With the aid of a calibration curve, immobilization degrees of

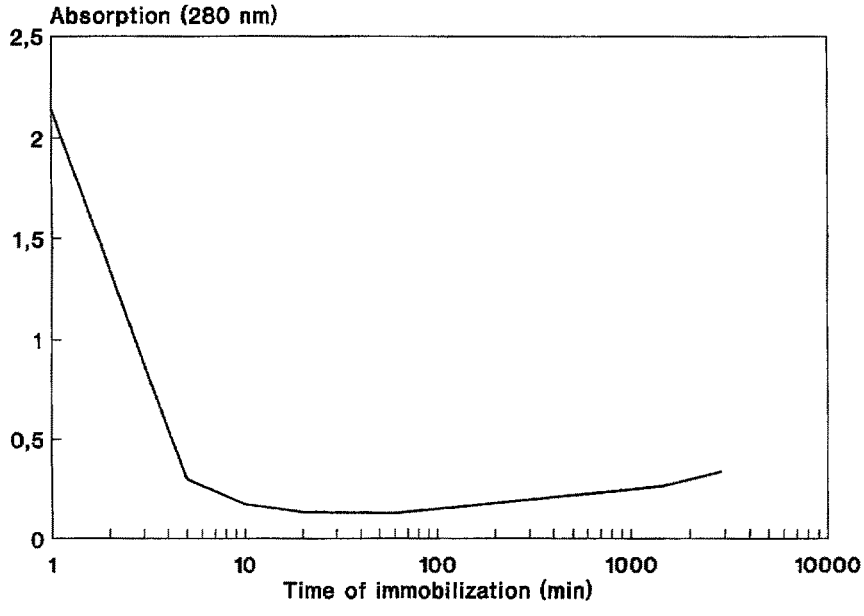


**Fig. 1** Absorption at $\lambda = 280$ nm of non-bound trypsin as a function of time of immobilization

about 85 % have been calculated after incubation for 48 h. Values of enzyme activity with BAEE substrate of 39.1 U/mg immobilized enzyme and 47.4 U/mg soluble enzyme were determined, respectively. Therefore, approximately 20 % of the immobilized trypsin is not active toward the substrate BAEE.

## Proteolysis of casein with immobilized trypsin

To characterize the proteolytic properties of the immobilized trypsin under standard conditions (see Material and Methods) the release of soluble amino-N from caseinate and the relative chain length of the caseinate peptides were determined as a function of the time of proteolysis (Fig. 2).

The content of soluble amino-N increased by approximately 200 µgN/ml after 4 h proteolysis while the relative chain length decreased by about 4 units to 6. Proteolysis was calculated to be 25 % less than that caused by homogeneous catalysis with the soluble enzyme (11). Furthermore, the soluble enzyme caused the release of 90 % of all measurable soluble amino-N within 10 min whereas the carrier-bound enzyme required more than 120 min.

Column chromatographic separation of the proteolysis products of caseinate after heterogeneous catalysis on an

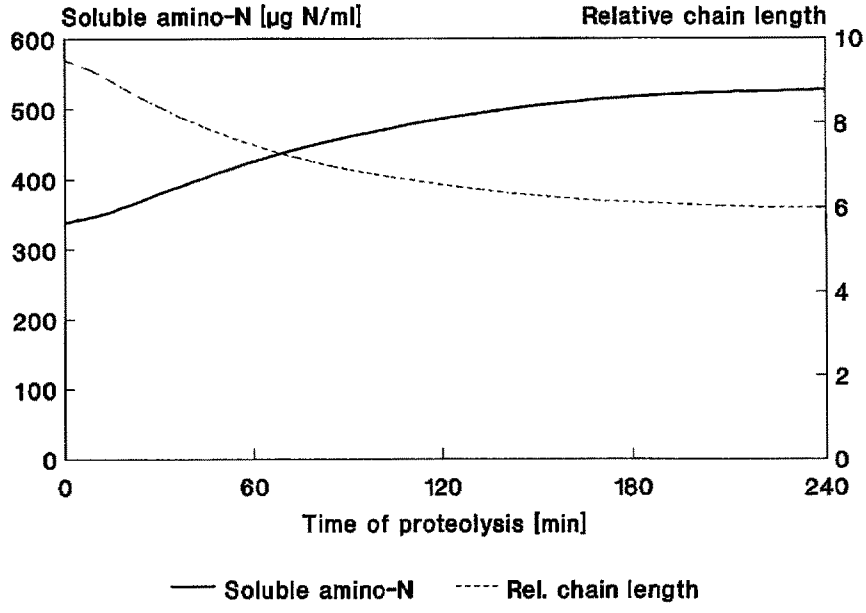


**Fig. 2** Soluble amino-N (µg N/ml) and relative chain length as a function of time of proteolysis of sodium caseinate under standard conditions

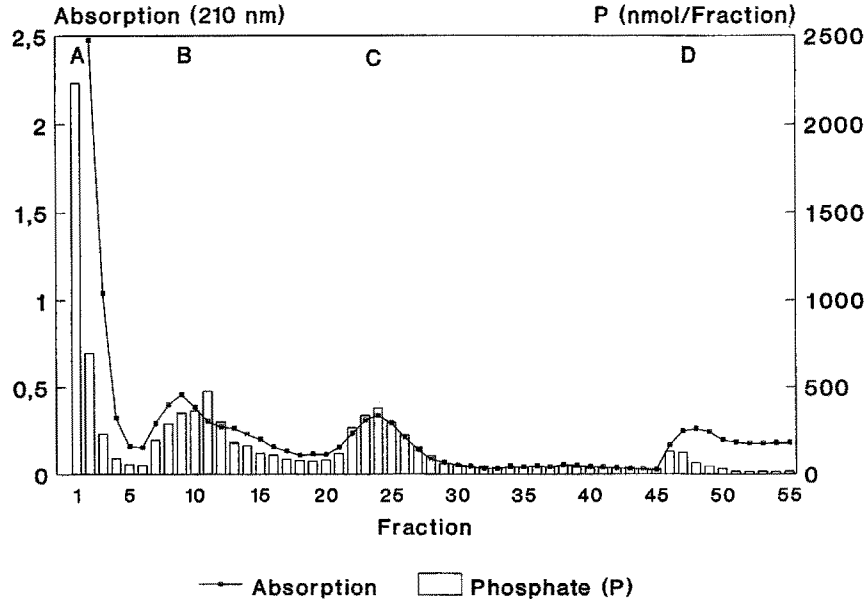


**Fig. 3** Elution profile (λ = 210 nm) and phosphorous content (nmol/fraction) following ion chromatographic separation of a proteolysate prepared from caseinate with immobilized trypsin under standard conditions

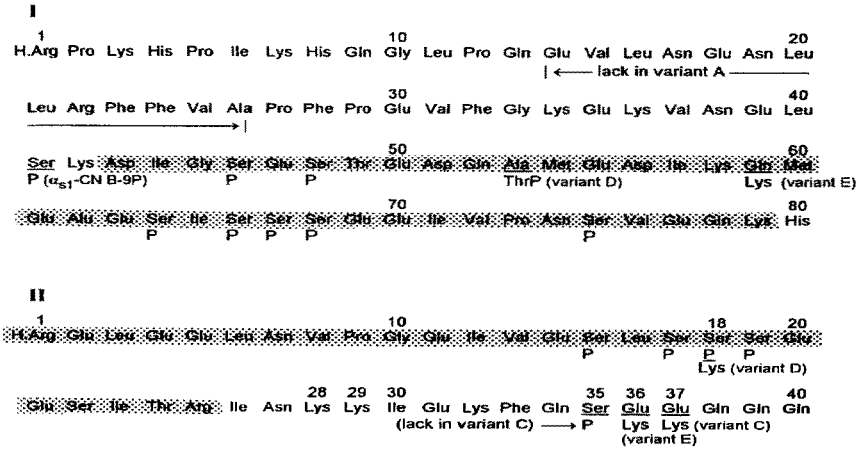


**Fig. 4** Phosphopeptide sequences (dotted regions) in the N-terminal primary structures[a] of bovine $\alpha_{s1}$-casein B-8P (I)[b] and β-casein $A^2$-5P (II)[b]

a) According to review articles of Eigel et al. (1984), Carles et al. (1988), and Ribadeau-Dumas (1988)

b) According to Meisel and Frister (1988), Meisel et al. (1991), Lorenzen et al. (1991), Meisel and Schlimme (1993)

| Caseinophosphopeptides | Regions in the primary structures of $\alpha_{s1}$- and β-casein [b] |
|---|---|
| $\alpha_{s1}$-casein-fragment | f43-58 |
| " | f59-79 |
| " | f66-74 |
| β-casein-fragment | f1-25 |

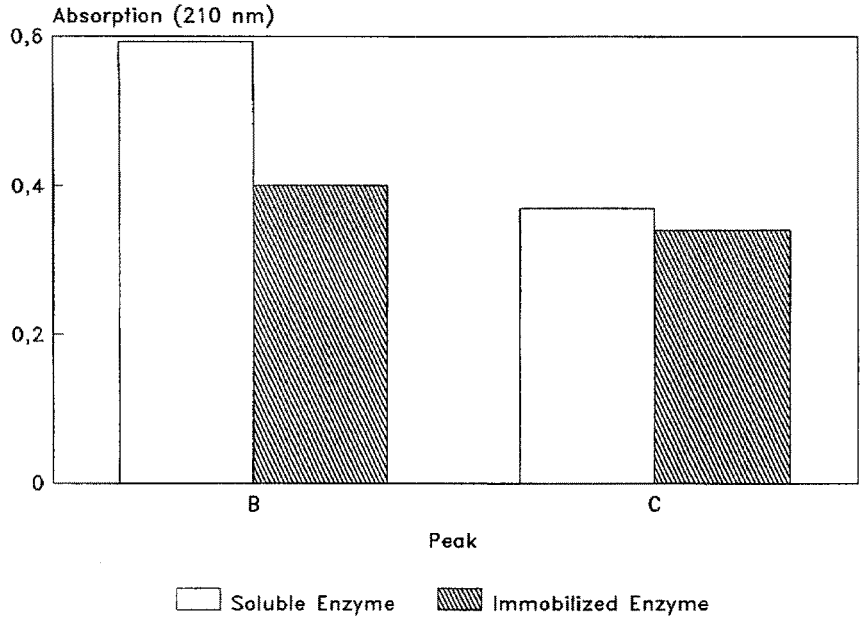


**Fig. 5** Peptide yields in peak B and peak C following ion chromatographic separation of proteolysates from caseinate prepared with soluble or immobilized trypsin under standard conditions

ion exchanger gave four peaks, labeled from A to D according to their sequence of occurrence (Fig. 3).

Peak A contains all proteolysis products that are not bound to the ion exchange resin at pH 2. CPPs from $\alpha_{s1}$-CN (43–58) and from β-CN (1–25) (Fig. 4) can be assigned as reference peptides for peaks B and C (14). The reference peptide for peak C is present in nearly the same concentration as after proteolysis with soluble enzyme, whereas the characteristic CPP of peak B occurs in markedly smaller concentrations than in the proteolysis carried out with free trypsin (Fig. 5); a lower calcium binding capacity was also established (16).

The CPP from $\alpha_{s1}$-CN (59–79) (Fig. 4), eluted in peak D when soluble trypsin is used, is absent in the chromatogram when the immobilized enzyme is used. This result was confirmed by analyzing the fractions 46–50 (peak D, Fig. 3) by HPLC after fractionation of the proteolysate by IE (figure not shown). This peptide may bind to the carrier by non-covalent interactions during proteolysis with immobilized trypsin which prevents it from passing into the filtrate used for the analysis. Reimerdes (21) demonstrated that immobilization of trypsin on oxirane beads increases the positive charge of the carrier-bound enzyme. The sequence 59–79 of $\alpha_{s1}$-CN has a strong

negative charge. However, washing the acrylic beads after proteolysis with sodium chloride (1 mol/l) and analyzing the wash by IE did not reveal phosphopeptide-rich fractions. This implies that the CPP from $\alpha_{s1}$-CN (59–79) is not formed during proteolysis, presumably because of steric hindrance – attributable to immobilization – at the catalytic center of the enzyme. Possible nutritional/physiologic consequences of the difference in composition of proteolysates after incubation with soluble or immobilized trypsin have not been investigated so far.

## Influence of the substrate concentration

For technological and economic reasons, the influence of the substrate concentration on the conversion rate of trypsin bound to the carrier has to be examined. Figure 6 shows the substrate saturation curve of the immobilized enzyme with casein as substrate (1–20 %, w/w).

The diagram shows that substrate saturation of the enzyme is attained at 15 % substrate concentration. Increasing the casein substrate to 20 % causes a decrease in the relative activity. The high viscosity of concentrated caseinate solutions may also influence the conversion rate of the immobilized enzyme. Caseinate solutions of 20 % have to be considered as sol, rather than as solution. The gradient of this curve tends to be less than that of substrate saturation curves found with soluble enzymes (6).

Because the rates of formation of soluble amino-N during the first 4 h of proteolysis are approximately linear (see Fig. 2), the amino-N values as a function of substrate concentration were incorporated into the reciprocal representation according to Lineweaver and Burk. As shown in Figure 7, the representation gives approximately a straight line.

Therefore, the action of the bound enzyme seems to accord with Michaelis-Menten kinetics. In the plot shown, the value for a substrate concentration of 1 % casein has been omitted. Figure 7 clearly shows that this value (see arrow) does not fit the linear relationship found for the other concentrations. Apparently, dilution of the substrate alters the proteolytic properties of carrier-bound trypsin.

Figure 8 shows the column chromatographic separation of the supernatants after proteolysis with 1–15 % protein solutions and isoelectric precipitation of the precipitable casein fragments.

The quantities of CPPs liberated at a substrate concentration of 1 % casein were either on or below the detection limit. At greater substrate concentrations, CPPs eluted in peaks B and C increase markedly with substrate concentration up to 15 %. Separation of the supernatants after proteolysis of 20 % casein substrate resulted again in smaller amounts of CPPs eluted (chromatogram not shown). Therefore, mean substrate concentrations of 5–10 % appear to be the most suitable for obtaining phosphopeptide-rich fractions with $\alpha_{s1}$-CN (43–58) and β-CN (1–25) as reference peptides from casein.

## Variation of the enzyme-substrate ratio

To characterize the influence of different amounts of enzyme on the composition of the proteolysate, enzyme-substrate ratios of 1/25, 1/50, 1/100, 1/200, 1/400 and 1/800 were prepared by decreasing the amount of immobilized enzyme at constant substrate concentration. Figure 9 shows the contents of soluble OPA-reactive amino-N (µg N/ml), which is proportional to the degree of hydrolysis, as a function of the enzyme-substrate ratio.

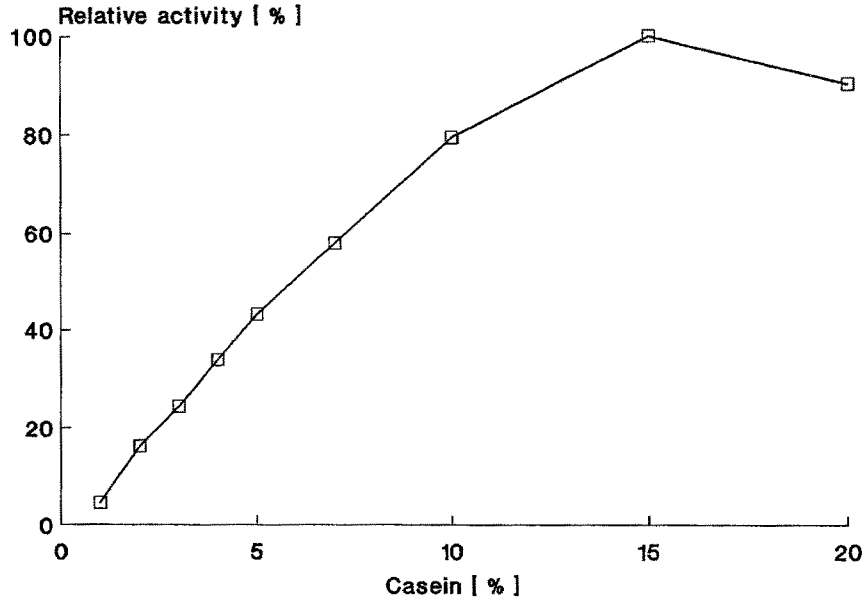


**Fig. 6** Relative enzymatic activity (%) of immobilized trypsin as a function of substrate concentration (proteolysates prepared under standard conditions with the exception of S)

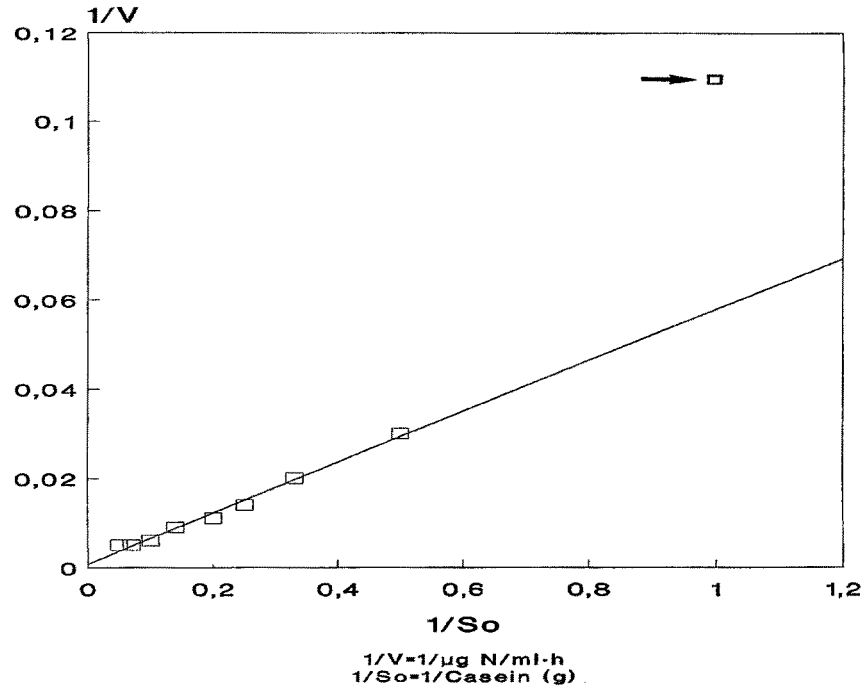


**Fig. 7** Lineweaver-Burk graph as a function of substrate concentration (1/V = 1/µg N/ml·h, 1/So = 1/Casein (g))

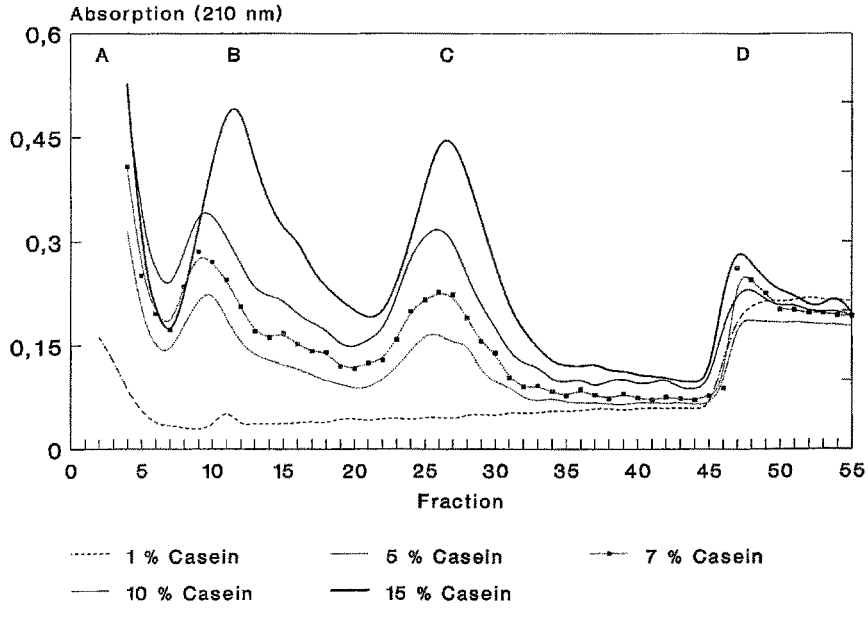


**Fig. 8** Elution profiles (λ = 210 nm) following ion chromatographic separation of proteolysates prepared at different substrate concentrations

Proteolysis increases exponentially with the amount of immobilized enzyme, in particular up to an E/S ratio of 1/100 (25 mg trypsin). However, at an E/S ratio of 1/50 (50 mg trypsin) the immobilized enzyme releases only two-thirds of the amount of soluble amino-N released by action of dissolved trypsin at an E/S ratio of 1/100 (11). Applying greater amounts of immobilized trypsin does not increase the relative activity significantly. This can be seen from the curve in Figure 9. Increasing the amount of enzyme to 100 mg (E/S = 1/25) results in lower relative activities. Figure 10 represents the results of column chromatographic separation of the filtrates obtained from proteolysis at different enzyme-substrate ratios.

It appears that the quantities of the CPPs eluted in peaks B and C decrease with enzyme proportion. Thus, peaks B and C are only weakly detectable after separation by ion exchange of proteolysates obtained at an E/S-ratio of 1/800. Separation of proteolysis products by HPLC revealed that proteolysates obtained at an E/S-ratio of 1/800 contain markedly smaller concentrations of hydrophobic peptides than proteolysates obtained at an E/S-ratio of 1/50 (10). This result suggests that at excess

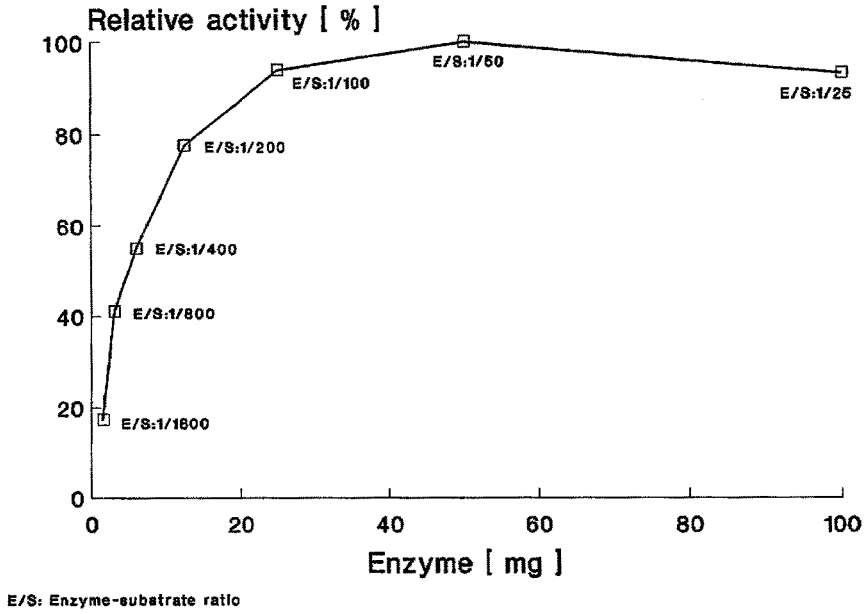


**Fig. 9** Relative enzymatic activity (%) of immobilized trypsin as a function of enzyme concentration (proteolysates prepared under standard conditions with the expection of E)

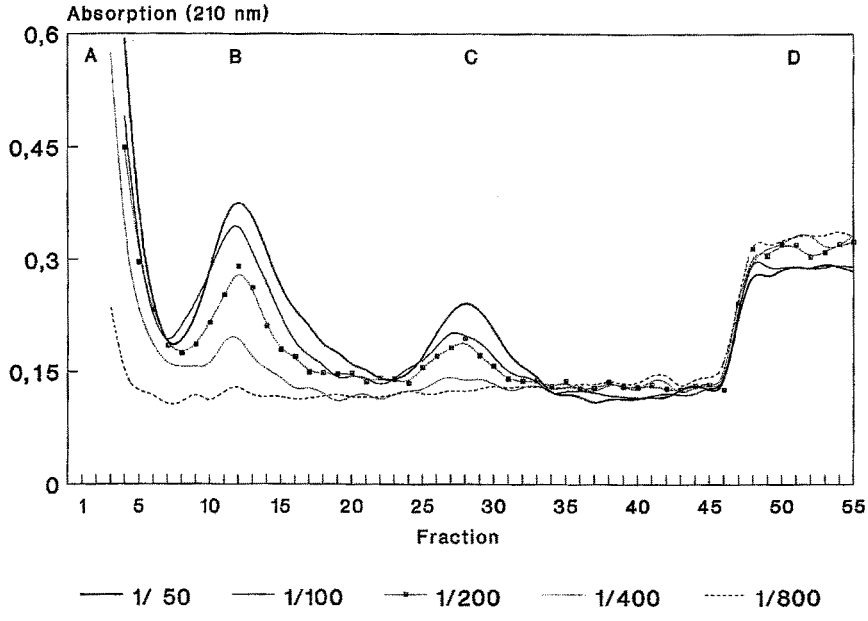


**Fig. 10** Elution profiles ($\lambda$ = 210 nm) following ion chromatographic separation of proteolysates prepared at varying enzyme-substrate ratios

substrate or lack of enzyme the mainly hydrophilic regions of the casein are preferentially hydrolyzed, or rather, that these regions are primarily accessible to tryptic proteolysis in aqueous systems. In addition, Danilenko et al. (3) describe that at small trypsin concentrations preferentially those peptide bonds are broken for which the enzyme exhibits the greater affinity. This finding indicates the limits, also from the economic point of view, to minimization of the use of enzyme, because lack of enzyme may lead to a qualitatively different composition of the resulting proteolysates. On the other hand, the differences in affinity of the enzyme for varying sequence areas of the substrate allow proteolysis control by varying the E/S ratio.

The incorporation of the conversion rates attained at varying enzyme-substrate ratios into the representation according to Eadie and Hofstee (Fig. 11) shows that the values for E/S ratios of 1/50–1/400 give approximately a straight line (broken line). However, the values for E/S ratios of 1/800 and 1/25 deviate from this linear behavior. Apparently, small and, especially, very large concentrations of immobilized enzyme lead to changed kinetic

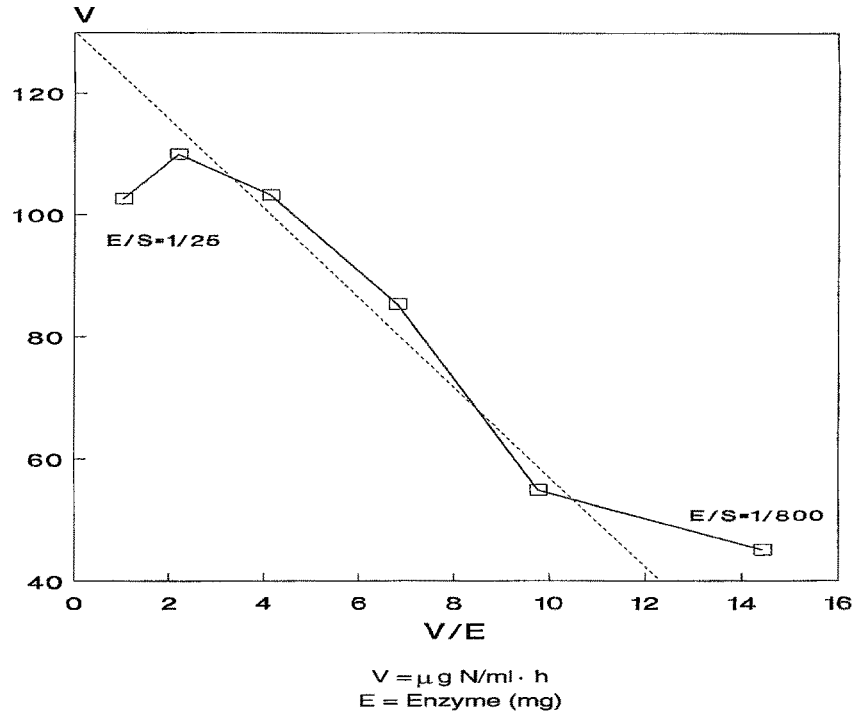


**Fig. 11.** Eadie-Hofstee Plot as a function of enzyme-substrate ratio (V = µg N/ml·h, E = enzyme (mg))

conditions during proteolysis. The same was true for small and partly also large substrate concentrations (see Fig. 7).

Therefore, mean E/S ratios of 1/50–1/100 represent optimal reaction conditions for proteolysis and, hence, for liberating CPPs.

## Influence of pH and temperature

The catalytic effect of immobilized enzymes and of the dissolved enzymes are strongly dependent on pH and temperature (6). Our studies have shown that stepwise increase of either pH from 7.8 to 9.3 or of temperature from 37 ° to 52 °C increases the conversion rates by at most 3 % and 13 %, respectively (figures not shown). Such small increases in enzyme activity at greater pH-values and temperatures alone do not justify modifying the initially used parameters of pH: 7.8 and temperature: 37 °C. However, column chromatographic separation of the proteolysates – e.g., at pH 8.8 (37 °C, Fig. 12) and 47 °C (pH 7.8, Fig. 13) –, clearly shows that the increase involves an overproportional liberation especially of the CPP β-CN (1–25). This can be seen from the elution chromatograms for the respective peaks C (pH 8.8 = + 47 %, 47 °C = + 89 %) in the figures. Although it is known that immobilization of enzymes leads to improved pH- and temperature stabilities (6, 8, 19), this should first be checked for the enzyme system at hand, before the changed values can be accepted as standard.

## Reuseability of the immobilized enzyme

Immobilization of enzymes allows the recovery of biocatalysts after the intended conversion of the substrate. The immobilized enzyme may be used repeatedly for basic research interest or for technical and economic reasons (6). In either case, the effect of recycling the immobilized enzyme on the proteolytic properties has to be determined. Mechanical forces that may occur in the stirred tank reactor as well as after enzymatic conversion during recovery and washing of the immobilized enzyme, may partially inactivate the enzyme or dissociate it from the carrier (25). Figure 14 shows the contents of soluble amino-N of a series of substrate solutions after treatment with the same immobilized enzyme (E/S = 1/50).

It was found that the degree of hydrolysis, measured as the content of soluble, OPA-reactive amino-N (µg N/ml), decreases with the number of repetitions. After the ninth repetition with the same immobilized enzyme the remaining proteolytic activity is still approximately 75 %. Similar results were obtained by Ueno and Morihara (24), who used trypsin immobilized on beaded agarose for semisynthesis of human insulin. They established approximately 80 % of the yield of the synthesis on average after nine successive runs. On the other hand, trypsin immobilized by adsorption onto chitin and used for proteolysis of gelatin retained only 50 % of its initial activity after the ninth repetition (17). Figure 15 represents the separation of the proteolysates by ion exchange chroma-

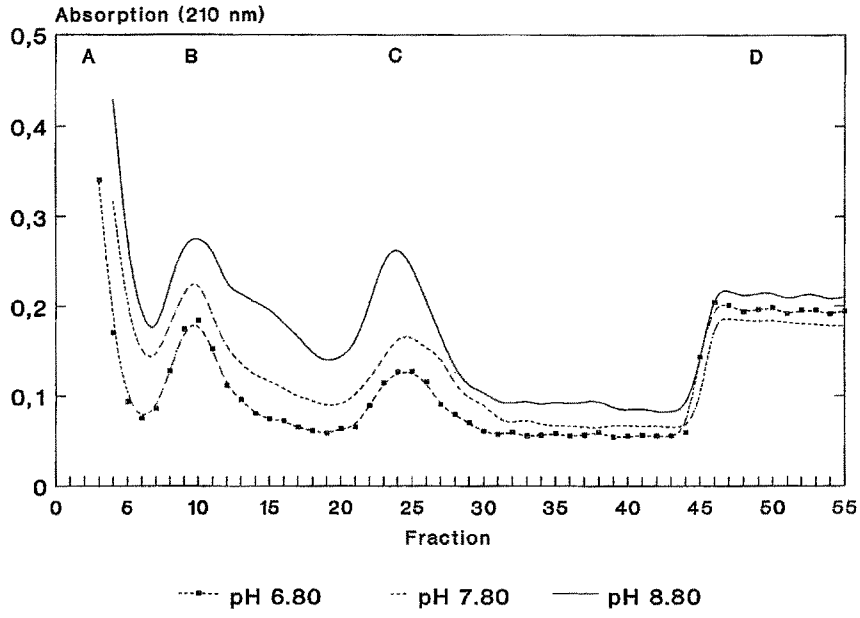


**Fig. 12** Elution profiles ($\lambda$ = 210 nm) following ion chromatographic separation of proteolysates prepared at different pH

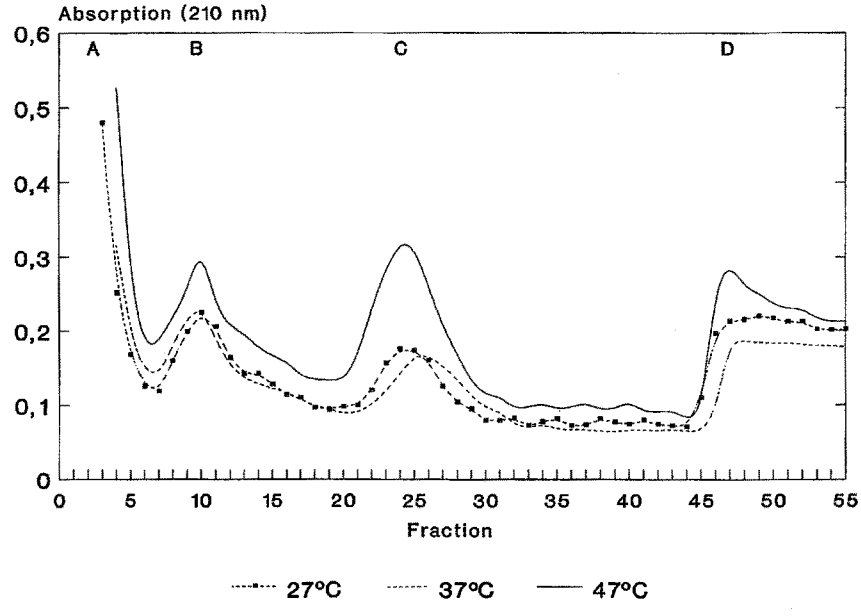


**Fig. 13** Elution profiles ($\lambda$ = 210 nm) following ion chromatographic separation of proteolysates prepared at different temperatures

tography after one, three, five, seven, and nine uses of the immobilized enzyme.

The yield of CPPs eluted in peaks B and C diminishes with the number of repeated runs with the immobilized enzyme confirming the results shown in Fig. 14. Of the CPPs that could be chromatographed after one single use of the immobilized enzyme, approximately 75 % are eluted in peaks B and C after the ninth repetition.

Reverse phase liquid chromatography analysis of the proteolysates after one and nine uses of the immobilized enzyme revealed – in contrast to the experiments with varying enzyme-substrate ratios – that reuse of the immobilized enzyme causes quantitative but no qualitative differences in the peptide pattern (10). As already described (25), the partial loss of proteolytic activity can be induced by mechanical energy. On the other hand, the mechanical energy breaks the acrylic beads after repeated use (see Fig. 16).

It cannot be excluded that the minor loss in proteolytic activity of about 25 % after nine uses is attributable to the breaking of the beads with liberation of the trypsin immobilized in the interior of the porous beads. Owing to their unsatisfactory mechanical stability, acrylic beads are suitable only to a limited extent for repeated use in

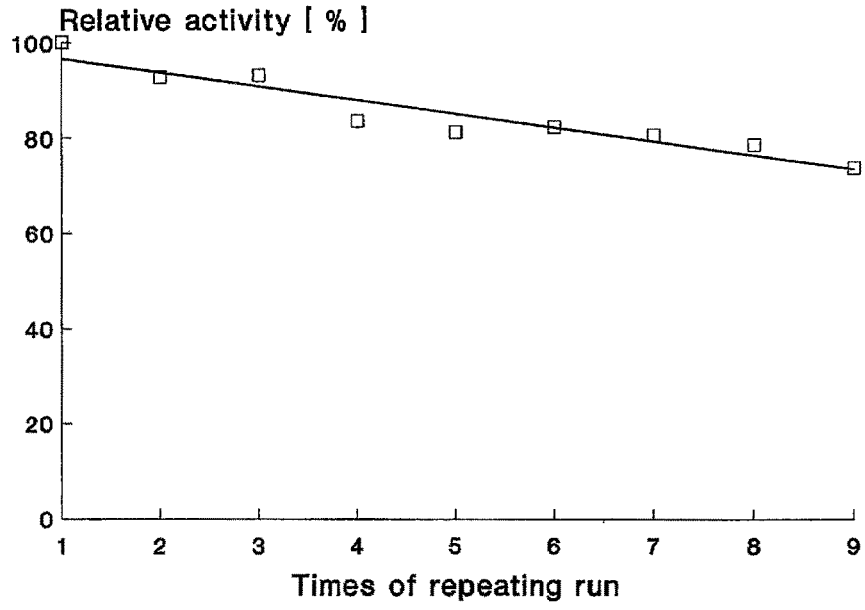


**Fig. 14** Relative enzymatic activity (%) of immobilized trypsin as a function of repeated uses (proteolysates prepared under standard conditions)

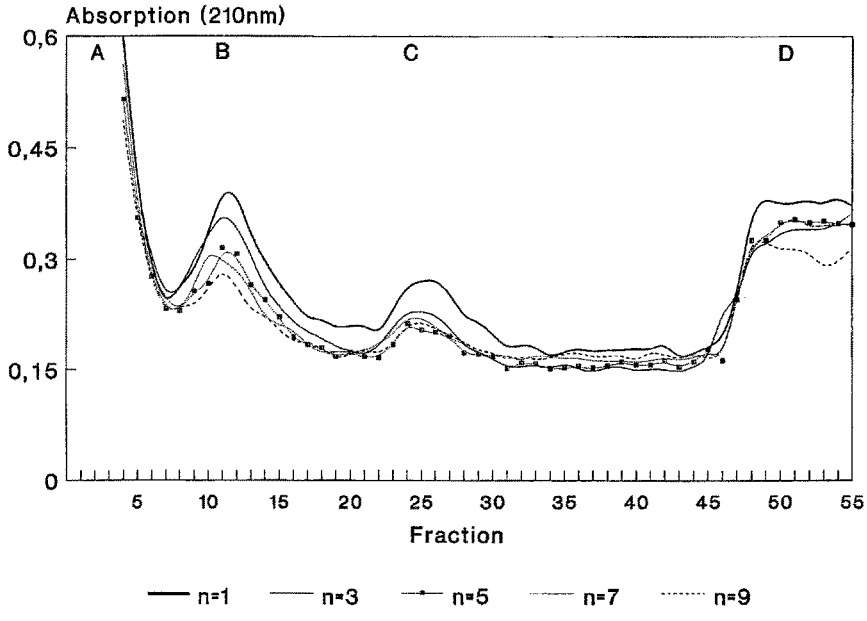


**Fig. 15** Elution profiles ($\lambda$ = 210 nm) following ion chromatographic separation of proteolyates after one–nine uses of immobilized enzyme

stirred tank reactors. In the present studies column reactors could not be applied, because during tryptic caseinolysis (9, 13) high-molecular-weight, plastein-like aggregates form that obstruct the column. Application of mechanical forces therefore, influences the proteolytic properties of the carrier-bound enzyme. Furthermore, casein fractions as well as proteolysis products of these proteins may cause intermediate- or end-product inhibition. Reimerdes (20), for example, established that highly hydrophobic proteolysis products of β-casein can cause inhibitory effects on the activity of immobilized trypsin.

## Conclusion

The studies have shown that trypsin can be covalently bound to oxirane-acrylic beads by means of a simple procedure. Use of immobilized enzyme led to 25 % less proteolysis than that observed with soluble enzyme under the same conditions. Furthermore, a modified peptide pattern of the resulting proteolysate was determined. A CPP from $\alpha_{s1}$-CN (59–79), which is detectable when soluble trypsin is used, is not found after proteolysis with the carrier-bound enzyme. Apparently, immobilization – possibly by steric hindrance at the active center – leads

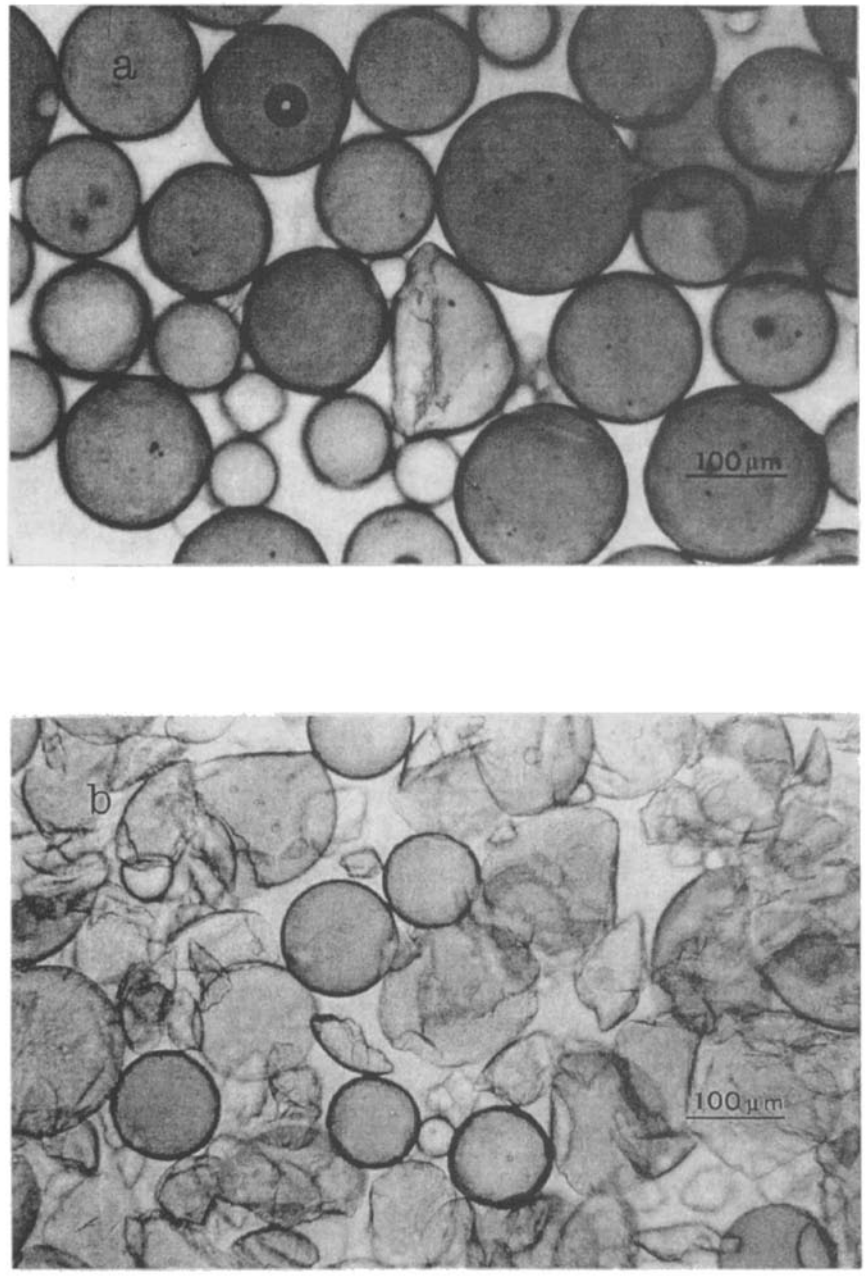


**Fig. 16** Light microscopic picture of the acrylic beads after a) one use and b) nine uses

to a changed cleavage specificity of the enzyme. Studies on the influence of the substrate concentration have clearly shown that casein contents of 5–10 % in the reaction mixture are most suited for obtaining phosphopeptide-rich fractions. Proteolysis of very small (S = 1 %) and partly also very large (S = 20 %) substrate concentrations is apparently subject to changed kinetic conditions. The same was true for small and large concentrations of immobilized enzyme in the reaction mixture. Compared with enzyme saturation (E/S = 1/50), lack of enzyme (E/S = 1/800) leads not only to a disproportional decrease in the proteolysis rate, but also to a different peptide composition of the proteolysates. Hence, the possibilities of minimizing the amount of enzyme used, which is economically desirable, are limited. On the other hand, control of proteolysis seems to be possible by varying E/S ratios. Increasing the pH and the temperature leads to both slightly larger conversion rates and an overproportional liberation of CPPs, in particular from β-casein. Repeated use of the immobilized trypsin resulted after nine repetitions in a 25 % smaller yield of CPPs. The peptide pattern of the proteolysate remained un-

changed. Because of their incomplete mechanical stability, however, acrylic beads appear to be suited only to a limited extent for repeated use in stirred tank reactors.

## Acknowledgement

We wish to express our thanks to Dr. David Tait for his helpful criticism in preparing this paper.
The study was supported by funds of the EU (E.C. reg. No. 663/88-6.4 and E.C. reg. No. 1001/90-11.2).

## References

1. Adamson N, Riley PF, Reynolds EC (1993) The analysis of multiple phosphoseryl-containing caseinpeptides using capillary zone electrophoresis. J Chromatogr 646:391–396
2. Carles C, Huet JC, Ribadeau-Dumas B (1988) A new strategy for primary structure determination of proteins: application to bovine β-casein. FEBS Lett 229:265–272
3. Danilenko AN, Dmitrochenko AP, Braudo EE, Bogomolov AA, Rozantsev EG (1993) Restricted enzymatic hydrolysis of legumin of broad beans by trypsin in concentrated solutions. Die Nahrung 37:46–52
4. Eigel WN, Butler JG, Ernststrom CA, Farrell jun HM, Harwalker VR, Jenness R, Whithey RMcL (1984) Nomenclature of Proteins of Cow's Milk. Fifth Revision. J Dairy Sci 67:599
5. Frister H, Meisel H, Schlimme E (1986) Modifizierte OPA-Methode zur Charakterisierung von Proteolyse-Produkten. Milchwissenschaft 41:483–487
6. Hartmeier W (1977) Immobilisierte Enzyme für die Lebensmitteltechnologie. Gordian 77:202–210
7. Kunst A (1992) Process to isolate phosphopeptides. European Patent Application EP 0476 199 A1
8. Leiva ML, Gekas V (1988) Application of immobilized enzymes in food processing. In: Robinson RK (ed) Developments in Food Microbiology 4. Elsevier Applied Science, London, New York, 121–147
9. Lorenzen PChr (1994) Untersuchungen zur Modifizierung von Casein mit Hilfe der Plasteinreaktion. Kieler Milchwirtschaftliche Forschungsberichte 46:179–190
10. Lorenzen PChr, Fischer H, Schlimme E (1994) Controlled in vitro proteolysis of casein using immobilized trypsin. Die Nahrung 38 (6):549–556
11. Lorenzen PChr, Pfaff K, Schlimme E (1991) In vitro-Proteolyse von Casein mit gelöstem und immobilisiertem Trypsin. Kieler Milchwirtschaftliche Forschungsberichte 43:189–198
12. Lorenzen PChr, Schlimme E (1990) Untersuchungen zur Enzyminduzierten Proteinaggregation (EIPA) an Natrium-Caseinat-Lösungen. Kieler Milchwirtschaftliche Forschungsberichte 42:553–563
13. Lorenzen PChr, Schlimme E (1992) The plastein reaction: properties in comparison with simple proteolysis. Milchwissenschaft 47:499–504
14. Meisel H, Behrens S, Schlimme E (1991) Calcium-Bindungsstudien an Phosphopeptidfraktionen aus der in vitro-Proteolyse von Casein. Kieler Milchwirtschaftliche Forschungsberichte 43:199–212
15. Meisel H, Frister H (1988) Chemical characterization of a caseinophosphopeptide isolated from in vivo digests of a casein diet. Biol Chem Hoppe-Seyler 369:1275–1279
16. Meisel H, Schlimme E (1993) Bindungsvermögen für Calcium und Eisen verschiedener Fraktionen aus der in vitro-Proteolyse von Casein. Kieler Milchwirtschaftliche Forschungsberichte 45:235–243
17. Nozawa Y, Matsushita T, Yamashina K, Higashide F (1982) Immobilization of trypsin on chitin and chitosan by solid-state mix-grinding. Biotechnol Bioeng 24:753–756
18. Prüfungsvorschrift: Code Nr. AZLD 308687, Merck, Darmstadt
19. Reddy CR, Reddy CR, Raghunath K, Joseph KT (1986) Immobilization of trypsin on alginic acid-poly (glycidyl methacrylate) graft copolymer. Biotechnol Bioeng 28:609–612
20. Reimerdes EH (1979) Model proteolysis of β-casein by immobilized trypsin. J Dairy Research 46:223–226
21. Reimerdes EH (1980) The effect of binding systems on immobilized enzyme suitability. Food Process Engin 2:69–80
22. Reynolds EC (1992) Production of phosphopeptides from casein. PCT. International Patent Application WO 92/18526
23. Ribadeau-Dumas B (1988) Structure and variability of milk-proteins. In: Milk proteins nutritional, clinical, functional, technological aspects (Barth CA, Schlimme E, eds) Steinkopff, Darmstadt and Springer, New York, pp 112–123
24. Ueno Y, Morihara K (1989) Use of immobilized trypsin for semisynthesis of human insulin. Biotechnol Bioeng 33:126–128
25. Uhlig H (1991) Enzyme arbeiten für uns. Carl Hanser Verlag, München, Wien