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Controlled in vitro proteolysis of casein using immobilized trypsin. Influence of variation of the enzyme-substrate ratio and the re-use of the immobilized enzyme on the preparation of phosphopeptide-rich fractions

P. CHR. LORENZEN, H. FISCHER and E. SCHLIMME

Federal Dairy Research Centre, Kiel, Federal Republic of Germany

Summary

Studies on in vitro proteolysis of casein using dissolved trypsin or covalently bound to oxirane beads have shown that immobilization leads to a change in the peptide pattern of the resulting proteolysate. Experiments on the variation of the enzyme-substrate ratio (E/S = 1/50, 1/100, 1/200, 1/400, 1/800) revealed that, compared with saturation (E/S = 1/50), lack of enzyme (E/S = 1/800) results not only in a disproportional decrease in the proteolysis rate, but leads additionally to a qualitatively differing peptide composition of the proteolysates. This variation, which appears, on the one hand, not acceptable in regard to a reproducible preparation of biologically active peptides is, on the other hand, a means of controlling proteolysis via the enzyme-substrate ratio. Re-use of the immobilized trypsin caused, after 9 repetitions, a loss in proteolytic activity and in phosphopeptide yields of approximately 25%, whilst the peptide pattern of the proteolysate remained qualitatively unchanged.

Zusammenfassung

Kontrollierte in vitro-Proteolyse von Casein mit immobilisiertem Trypsin. Einfluß der Variation des Enzym-Substrat Verhältnisses und der Mehrfachnutzung des Immobilisats auf die Gewinnung phosphopeptidreicher Fraktionen

Untersuchungen zur in vitro-Proteolyse von Casein mit gelöstem oder kovalent an Oxiranperlen gebundenem Trypsin haben gezeigt, daß die Immobilisierung zu einem veränderten Peptidmuster des resultierenden Proteolysates führt. Versuchsreihen zur Variation des Enzym-Substrat Verhältnisses (E/S = 1/50, 1/100, 1/200, 1/400, 1/800) haben deutlich gemacht, daß Enzymmangel (E/S = 1/800) – im Vergleich zur Enzymsättigung (E/S = 1/50) – nicht nur zu einer unproportionalen Abnahme der Proteolyserate, sondern darüberhinaus zu einer qualitativ differierenden Peptidzusammensetzung der Proteolysate führt. Diese Variation, die einerseits im Hinblick auf eine reproduzierbare Gewinnung biologisch aktiver Peptide nicht akzeptabel erscheint, bietet andererseits eine Steuerungsmöglichkeit der Proteolyse über das Enzym-Substrat Verhältnis. Die Mehrfachnutzung des immobilisierten Trypsins führte nach neunfacher Anwendung zu einem Verlust der proteolytischen Aktivität und der Ausbeute an Phosphopeptiden von ca. 25%, das Peptidmuster des Proteolysats blieb aber qualitativ unverändert.

Introduction

For liberating phosphopeptide-rich sequences from the primary structure of casein fractions normally soluble proteinases are used [1, 2], which can either not or only partially [3] be recovered after hydrolysis and represent, hence, a considerable cost factor in the development of enzyme-controlled proteolysis procedures. With the same efficiency and specificity the use of carrier-bound proteinases would be less costly [4].

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Own studies on in vitro proteolysis of casein by dissolved trypsin or immobilized on oxirane beads [5] have, however, shown that the use of the immobilized enzyme leads, under comparable conditions, to significant lower degrees of hydrolysis and longer chain lengths of the peptide fragments. Separation of the proteolysis products via ion exchanger has, further, shown that when using the dissolved enzyme a caseinophosphopeptide occurs, which is not eluted, if the immobilized enzyme is used. It is possible that this peptide is attached to the carrier by noncovalent interactions during proteolysis with immobilized trypsin and has not passed into the filtrate used for the analysis. REIMERDES [6] demonstrated that immobilization of trypsin on oxirane beads leads to an increase in the positive charge of the immobilized enzyme, whereas phosphopeptides are highly negatively charged. However, more detailed studies [7] revealed that no phosphopeptide-rich fractions are bound to the carrier. This result suggests that the caseinophosphopeptide is not liberated because of steric hindrance – attributable to immobilization – at the catalytic centre of the enzyme.

Besides the characterization of possible changes in peptide pattern attributable to immobilization one has, when immobilized biocatalysts are used, to examine to what extent the proteolytic properties are influenced by the variation of the enzyme-substrate ratio and the re-use of the immobilized enzyme. A change of the enzyme-substrate ratio can, for instance, lead to a variation of the type of proteolysis ("one-by-one" or "zipper" mechanism) and the composition of the proteolysate [8, 9]. As regards the re-use of the immobilized enzyme one has to verify in how far the proteolytic activity is retained as a function of the number of repetitions. Here the literature indicates values between 30–90% after 9 applications depending on the immobilization technique and serine proteinase [10–13].

Material and methods

Oxirane beads (Eupergit® C, Röhm Pharma GmbH, Weiterstadt, epoxide content approximately 600 µmol/g dry beads) were used as support material for immobilizing trypsin.

9 g of dry oxirane beads were washed on a D4-glass frit using 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. For immobilization the trypsin buffer solution was quantitatively transferred to the washed oxirane beads; conversion was performed at room temperature for 48 h. The immobilization process was monitored photometrically (λ = 280 nm) by measuring the non bound trypsin. After 48 h the immobilized enzyme was 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 immobilization mixture after 48 h (28.4 mg). The difference of 151.7 mg trypsin was bound to 31.5 g of wet oxirane beads (swelling capacity is 2.5 ml of water per gram dry beads). To attain e.g. 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.

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.

Proteolysis of the sodium caseinate solutions was performed using the pH-stat method [14]:

Protein content (demineralized water):	5.0% (w/w)
Temperature:	37 °C
pH (1 mol/l NaOH):	7.8
Proteolysis time:	240 min

After sedimentation of the beads immobilized with trypsin the supernatant was decanted, subjected to acid precipitation at pH 4.6 (1 mol/l HCl) and the soluble proteolysis products were separated by filtration (filter: 595 1/2) [5].

The supernatants obtained by isoelectric precipitation (pH 4.6) of the proteolysis products were analysed for their peptide pattern using ion exchange chromatography according to the method described by MEISEL et al. [15]:

Column: Height 18 cm, diameter 0.9 cm
Resin: Dowex ion exchange resin, p.A., type 1 × 2, 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 λ = 210 nm were performed using a photometer Shimadzu UV-120-02, Shimadzu, Düsseldorf.

For characterizing the proteolysis products the modified OPA-method [16] was used. Separation of the peptides by reserve phase liquid chromatography was carried out as described by MEISEL et al. [17].

All results are based on duplicate determinations of 2 series of independent experiments.

Results and discussion

Variation of the enzyme-substrate ratio

For characterizing the influence of different amounts of enzyme used on the resulting composition of the proteolysates enzyme-substrate ratios of 1/50, 1/100, 1/200, 1/400 and 1/800 were realized by a corresponding decrease in the proportion of immobilized enzyme.

Fig. 1 shows the contents of soluble OPA-reactive amino-N (μg N/ml) as a function of the enzyme-substrate ratio.

If the content of soluble amino-N is considered the measure of the degree of proteolysis, an exponential increase in the degree of hydrolysis is observed with increasing enzyme proportion, in particular up to an E/S ratio of 1/100. Compared with proteolysis using dissolved trypsin (E/S = 1/100) [5] only approximately two thirds of the content of soluble amino-N can, however, be determined when using the immobilized enzyme, even at an E/S ratio of 1/50. Application of still higher amounts of immobilized trypsin appears not very useful, because from the curve in Fig. 1 it can be seen that with an enzyme-substrate ratio of 1/50 (0.02) the majority of the substrate has formed an enzyme-substrate complex so

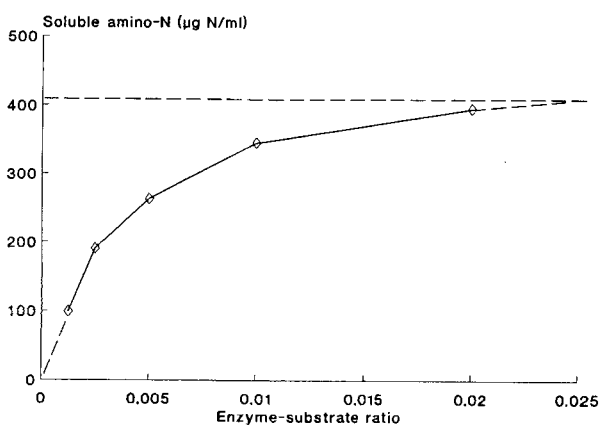

Fig. 1
Soluble amino-N (μg N/ml) as a function of the enzyme-substrate ratio

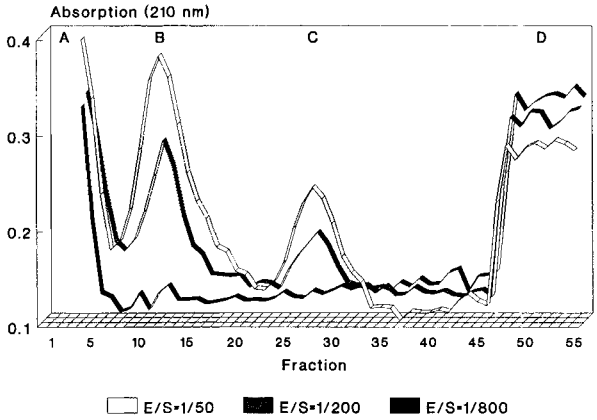

Fig. 2
Ion chromatographic separation of the filtrates after proteolysis at varying enzyme-substrate ratios

that not relevant increases in the degree of proteolysis are to be expected with higher enzyme quantities.

Fig. 2 represents the result of column chromatographic separation of the filtrates via ion exchanger after proteolysis at enzyme-substrate ratios of 1/50, 1/200 and 1/800.

It appears, that with decreasing enzyme proportion lower quantities of the caseino-phosphopeptides eluted in peaks B and C are liberated. With proteolysates obtained using an E/S ratio of 1/800 only signs of peaks B and C are recognisable after separation via ion exchanger.

From the HPLC-diagrams of proteolysis products obtained using E/S-ratios of 1/50 and 1/800 it appears that the curves take rather varying courses (Fig. 3).

Only up to a retention time of approximately 22 min, during which the mainly hydrophilic peptides are eluted, there is a certain qualitative identity of the chromatograms. Retention times of 23 – 35 min (elution of mainly hydrophobic peptides) give qualitatively and mainly quantitatively differing diagrams with a markedly lower concentration of hydrophobic

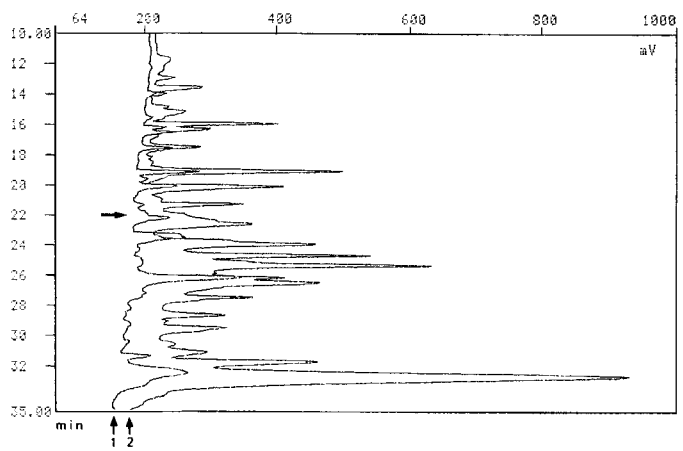

Fig. 3
Reverse-phase liquid chromatographic separation of the filtrates after proteolysis at enzyme-substrate ratios of 1/50 (2) or 1/800 (1)

peptides in the proteolysate obtained using an E/S ratio of 1/800. This result suggests that the mainly hydrophilic areas of the casein are, with excess substrate or lack of enzyme, preferentially hydrolysed or rather that these areas with a tryptic proteolysis in hydrous systems are primarily accessible. DANILENKO et al. [9] describe also that with low trypsin concentrations preferentially peptide bonds are broken down for which the enzyme exhibits the higher affinity. This finding is indicative of the limits existing, also from the economic point of view, as regards a minimization of the use of enzyme, because lack of enzyme may lead to a qualitatively differing composition of the resulting proteolysates. On the other hand, the differences in affinity of the enzyme for varying sequence areas of the substrate is also a means of proteolysis control via the E/S ratio.

Re-use of the immobilized enzyme

Immobilization of enzymes which enables a recovery of biocatalysts after the intended conversion of the substrate and the repeated use of the immobilized enzyme is performed in the interest of basic research and for technical-economic reasons [18]. Besides the characterization of varying enzyme-substrate ratios it has to be verified to what extent the re-use of the immobilized enzyme influences the proteolytic properties. The influence of mechanical forces as they may occur during incubation in the stirred tank reactor and after enzymatic conversion by recovery and washing of the immobilized enzyme, may lead to partial inactivation or dissociation of the enzyme from the carrier [19].

In the following, it is described how these facts present themselves in the case of carrier-bound casein proteolysis with trypsin. Fig. 4 shows the contents of soluble amino-N as a function of the times of repeating run using the same immobilized enzyme (E/S = 1/50).

It was found that the degree of hydrolysis, measured via the content of soluble, OPA-reactive amino-N (μg N/ml), decreases with increasing numbers of repetitions. After the ninth application of the same immobilized enzyme the remaining proteolytic activity reaches still approximately 75%.

Similar results were obtained by UENO et al. [12], who used immobilized trypsin attached to beaded agarose for semisynthesis of human insulin and have established, after 9 successive runs, approximately 80% of the yield of the synthesis, on the average. On the other hand,

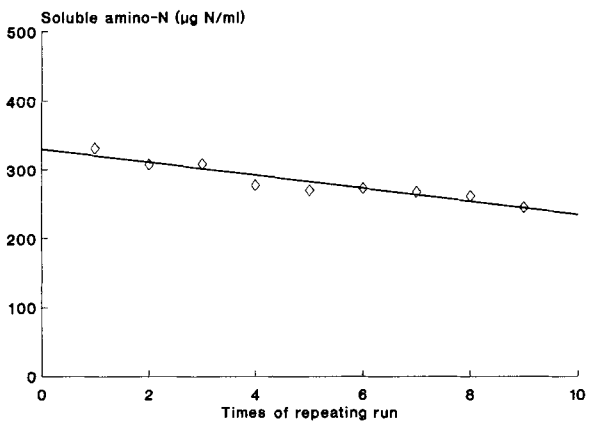

Fig. 4
Soluble amino-N (μg N/ml) as a function of the number of uses of the immobilized enzyme

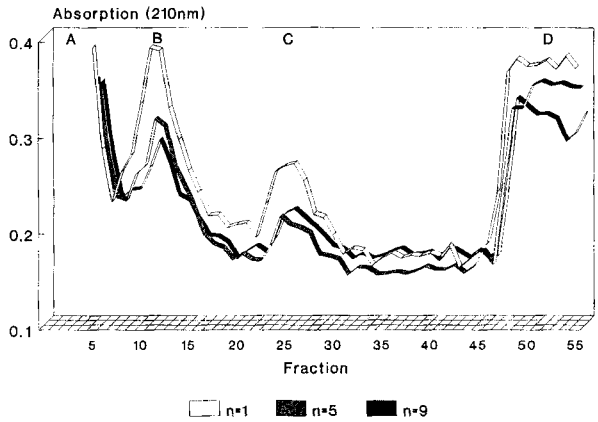

Fig. 5
Ion chromatographic separation of the filtrates after 1, 5, and 9 uses of the immobilized enzyme

trypsin immobilized by adsorption onto chitin and used for proteolysis of gelatine exhibited only 50% of its initial activity after the ninth repetition [10].

Fig. 5 represents the separation of the proteolysates using ion exchange chromatography after 1, 5 and 9 uses of the immobilized enzyme.

Similarly as Fig. 4, Fig. 5 revealed that with increasing numbers of applications of the immobilized enzyme the degree of proteolysis is decreasing and, hence, the yield of phosphopeptides, eluted in peaks B and C, diminishes. Measured via the extinctions approximately 75% of the phosphopeptides, which could be chromatographed after one single use of the immobilized enzyme, are still eluted in peaks B and C after the ninth repetition.

From Figs. 6 and 7 it can be seen that – contrary to the experiments with varying enzyme-substrate ratios – the re-use of the immobilized enzyme leads to quantitative, however not qualitative differences in the peptide pattern of the proteolysates after 1 and 9 uses of the immobilized enzyme.

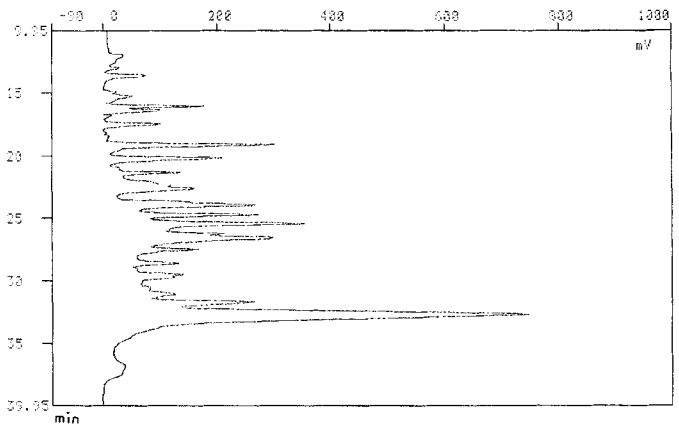

Fig. 6
Reverse-phase liquid chromatographic separation of the filtrate after 1 single use of the immobilized enzyme

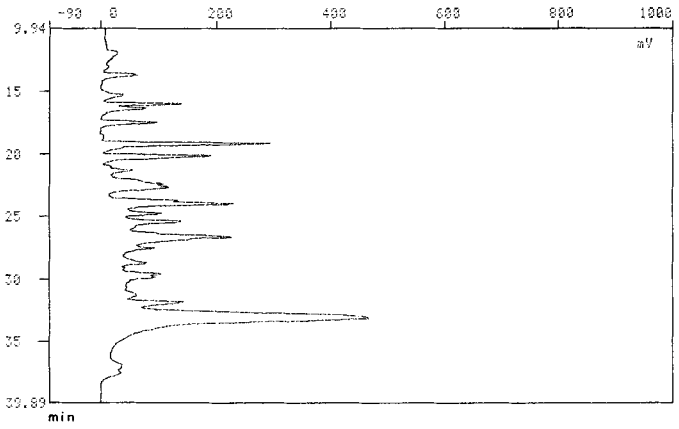

Fig. 7
Reverse-phase liquid chromatographic separation of the filtrate after 9 uses of the immobilized enzyme

As already described, the partial loss of proteolytic activity can be brought about under the influence of mechanical energy. Further it might be possible that casein fractions as well as proteolysis products of these proteins produce an intermediate- or end-product inhibition. So, for instance, REIMERDES [20] established that highly hydrophilic proteolysis products of beta-casein can cause inhibitory effects on the activity of immobilized trypsin. Whether in connection with the experiments in hand enzyme inhibition occurs is to be elucidated in further studies.

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Dr. P. CHR. LORENZEN, Federal Dairy Research Centre, Kiel, Institute for Chemistry and Physics, P.O. Box 6069, D-24121 Kiel, Federal Republic of Germany

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