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Quantitative comparison of casein and rapeseed proteolysis by pancreatin

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Summary

The problem of quantitative comparison of kinetic curves was solved for casein and rapeseed pancreatin hydrolysis in a membrane reactor, which ensured the measurement of proteolysis kinetics for the products with a molecular weight of less than 1000. Coordinates were derived which provided good linearization of kinetic curves and the determination of relative rate constants irrespective of reagent concentrations, E_0/S_0 ratio and time intervals of kinetic measurements. When the relative rate constants of the release of the individual amino acid residues in the low-weight proteolysis products were compared, trypsin-dependent constants (for Lys and Arg residues) were found to be two times less for rapeseed than for casein, and chymotrypsin-dependent constants (for Tyr and Phe residues) were approximately 1.3 times higher for rapeseed than for casein. Statistical analysis demonstrated that the distribution of constants was narrower for rapeseed than for casein. Differences between target (Arg, Lys, Tyr and Phe) and non-target constants of release in the form of peptides and free amino acids, or in the form of free amino acids only, were attributed to the differences in the peptide bond masking for casein and rapeseed proteins. Computer simulation of proteolysis kinetics was performed by PROTEOLYSIS program package to confirm the dependence of rate constant distribution on the state of masking.

Introduction

Proteolysis in open reactors with the continuous removal of products is of great interest for the studies of the digestion process. Since SAVOIE and GAUTHIER [1] developed the "digestion cell" reactor, which modeled the pancreatic stage of digestion, the digestibility of several proteins has been determined as function of proteolysis time by means of this technique [2, 3]. Another type of reactor, modeling the peptic stage of digestion, was also developed [4, 5]. The comparison of the digestibility values obtained for different substrates *in vitro* for any fixed time is questionable, because these values depend not only on the real parameters of enzyme-substrate interaction (such as k_{cat} and K_M, the MICHAELIS's constants) but also on the initial reagent concentrations (S_0, E_0 and S_0/E_0) and proteolysis time, which are arbitrary and whose influence on the outcome of the proteolysis could lead to wrong conclusions about the susceptibility of different proteins to digestion. The objective comparison of the *in vitro* digestibilities of different substrates independently of the conditions of the kinetic measurements seems to be possible only by the comparison of kinetic parameters, which describe proteolysis curves as a whole.

Meanwhile, it is difficult to obtain quantitative parameters of proteolysis kinetics even in closed reactors without removal of hydrolysis products [6]. Proteolysis is a polysubstrate process, involving hydrolysis of peptide bonds of different specificity, which is complicated by masking of these bonds, as well as by enzyme inhibition and inactivation [7]. Among the literature on the proteolysis parameter determination [8, 9, 10–12] the last series describes this process in the terms of functions, instead of rate constants. These functions, at an equivalent degree of peptide bond hydrolysis, can be compared for different substrates [12]. However, this approach needs to be modified for the description of proteolysis in an open reactor such as the digestion cell [11], which is the result of simultaneous action of various proteolytic enzymes under continuous removal of digestion products. In this case, it seems more appropriate to consider distinct rate constants for each type of amino acid. Each rate constant can characterize the kinetics of the appearance outside the open reactor of a given type of amino acid residue in the form of peptides or free amino acid. To achieve the goal of substrate comparison, one can calculate ratios between absolute constants for each of substrates, because in this case the common factor

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in the absolute constants will be canceled. For given protein substrate, part of the common factor in absolute constants is attributed to some accidental factors like presence of inhibitors etc. Introducing of relative constants will simplify the kinetic description of such complicated phenomena like digestion, as well as the comparison of protein substrates.

Casein is known to be easily hydrolysed by proteolytic enzymes because of the conformational flexibility and the presence of accessible peptide bonds for proteolytic action. Target amino acid residues of chymotrypsin and trypsin are liberated quickly and are liable to appear first outside the membrane reactor. In contrast, plant proteins, as well known, are more resistant to proteolytic action as the oligomeric proteins, consisting of compact extensively disulfide-linked polypeptide subunits, and also due to the presence of inhibitors. For example, amino acids of rapeseed proteins are liberated from digestion cell more slowly and gradually [2, 13].

As substrates for proteolysis, casein and rapeseed differ in the state of demasking (masking), the extent of peptide bonds which are susceptible to enzyme attack. The different states of masking for different substrates are attributed to various factors including the burying of intrinsic peptide bonds inside the protein globule, intermolecular aggregation, isolation of part of peptide bonds as the result of incomplete solubilization, etc.

Proteolysis is the two-stage process, including the demasking of masked peptide bonds B_m on the first stage and the hydrolysis of demasked bonds B_d on the second stage [10]:

$$B_m \xrightarrow{k_d} B_d \xrightarrow{k_h} N,$$

where k_d is the rate constant of demasking, k_h is the rate constant of hydrolysis of demasked bonds, and N is the concentration of amino nitrogen, the chemical outcome of the reaction. Ratio k_d/k_h is the kinetic parameter which describes the influence of demasking stage on the whole proteolysis process. Low value of k_d/k_h was attributed to the 'one-by-one' mechanism, and high value of k_d/k_h indicated the proteolysis of the 'zipper' type [10, 14]. This parameter was measured for proteolysis of milk whey proteins [14].

An extensive comparison measured at numerous intervals of proteolysis showed a positive correlation between the composition of digestion products obtained from *in vitro* proteolysis of different proteins and the amino acid composition of the portal blood of animals fed by these proteins [15]. However, this *in vivo* characterization was lengthy and tedious, and cannot be repeated routinely.

The aim of the present work was to elaborate a method for the treatment of kinetic curves obtained by digestion cell technique, to determine relative rate constants and to compare them for casein and rapeseed. Experimental data were obtained from three different sets of experiments which were performed over five years.

Another task of the study was to simulate amino acid composition of the low-weight proteolysis products in the course of proteolysis of proteins with different states of masking. This computer simulation was designed to examine the relationship between masking and distribution of rate constants.

Materials and methods

In vitro digestion

In vitro digestion of casein (Na caseinate, UCCCP, France, $N \times 6.25 = 86.8\%$) and rapeseed (concentrate, CETIOM, France, $N \times 6.25 = 52\%$) was carried out in a digestion cell (pancreatic stage) with preliminary peptic proteolysis in a closed reactor [1].

Pepsin (EC 3.4.23.1, hog stomach mucus) was purchased from Sigma Chemical Company, St. Louis, MO. The pepsin activity was 3.2×10^3 units/mg protein [16]. Pancreatin (hog pancreas, $5\times$) was obtained from ICN Nutritional Biochemical, Montreal, Canada. Trypsin and chymotrypsin activities were 6.34 TAME units/mg and 5.88 ATEE units/mg respectiely [17].

The conditions for the peptic stage were exactly the same in all experiments. Proteins (40 mg) were suspended in 16 ml 0.1 M HCl (pH 1.9) and digested with 1 ml of pepsin (1 mg/ml) for 30 min. This pre-digested material was then subjected to pancreatin action. The constant conditions for this stage were: dialysis bag (Spectra Por 6, MWCO of 1000 daltons, Spectrum Medical ind., L.A., CA), temperature 37 °C, flow rate 1.6 ml/min of 0.01 M phosphate buffer (pH 7.5) to collect digestion products from digestion cell. The variable conditions in different sets of experiments were:

Set A, carried out by SAVOIE et al. [2]: 1 ml of 10 mg/ml pancreatin solution was added to the digestion cell. E_0/S_0 ratio was 1:25. Analyses were performed after 3, 6, 9, 12 and 15 h of proteolysis. The number of repetitions was 4.

Set B, varried out by VALETTE et al. [18]: The E_0/S_0 ratio was the same (1:25). Analyses were performed after 1, 2, 3, 6 and 24 h of proteolysis. The experiment was repeated 4 times.

Set C, carried out by SAVOIE et al. [19]: 0.5 ml of 10 mg/ml enzyme solution was added to the digestion cell (E_0/S_0 ratio was 1:50). The times of sampling were: 0.5, 1, 2, 3, 12 and 24 h. The experiment was repeated 3 times.

Amino acid digestibility (AAD) for each sampling interval was calculated on the basis of amino acid composition (AA) of low-weight proteolysis products as follows:

$$\text{AAD} = \frac{\text{AA in dialysate (mg)}}{\text{AA in protein sample (mg)}}. \quad (1)$$

Amino acid analysis was performed as described in [3].

Method of constant determination

Relative rate constants k_i were measured as normalized rate parameters with which amino acid residues i go through the membrane. Evaluation of k_i was performed by means of following equation:

$$k_i = \frac{\ln(1 - D_i)}{\ln(1 - D)}, \quad (2)$$

where D_i is the AAD evaluated with eq. (1), $D = \Sigma D_i w_i$ is the total degree of release of all peptides and amino acids in dialysate, and w_i is the molar fraction of each amino acid in substrate.

The same eq. (2) is also valid for relative constant evaluation of free amino acid release from digestion cell (k_i^{aa}). In this case, AA in dialysate (eq. 1) refers to the fraction of free amino acids. The kinetic data concerning release of free amino acids only were taken from [2].

Statistical analysis

The program for the coordinate transformation was written with Statistical Analysis System language (SAS Institute, Carry, NC). Differences between means were assessed by DUNCAN's test. GLM and FACTOR procedure were performed with the SAS.

Computer simulation of proteolysis

Simulation of proteolysis kinetics was performed by means of PC software PROTEOLYSIS (Foodinform Ltd., Moscow, Russia) [6].

PROTEOLYSIS program automatically estimates all necessary kinetic parameters for the set of differential equations which describe full kinetics for all possible components. Specificity parameters and K_M for chosen enzymes are from PROTEOLYSIS database. Special subroutine helps user to define amino acid sequence in the studied substrate. The input data consist of E_0, S_0, parameter of demasking and proteolysis time t. After numerical integration of differential equation set, the PROTEOLYSIS program represents kinetic output data in graphic form. This program was written in C.

Results and discussion

For casein and rapeseed proteolysis in the digestion cell, three sets of kinetic data were obtained for conditions denoted as A, B and C in the terms of AAD vs. time of proteolysis. Description of A, B and C conditions are given in the experimental part. As an example, the typical non-linear curves are presented in Fig. 1 for casein proteolysis (experiment C). The data for each protein, each amino acid (type I) and each condition were represented in the new coordinates expressed as $y = -1/\ln(1 - D_i)$ and $x = -1/\ln(1 - D)$ (according to eq. (2)). In these coordinates (Fig. 2), proportional relationship takes place, and only one parameter, the slope $1/k_i$, characterizes the rela-

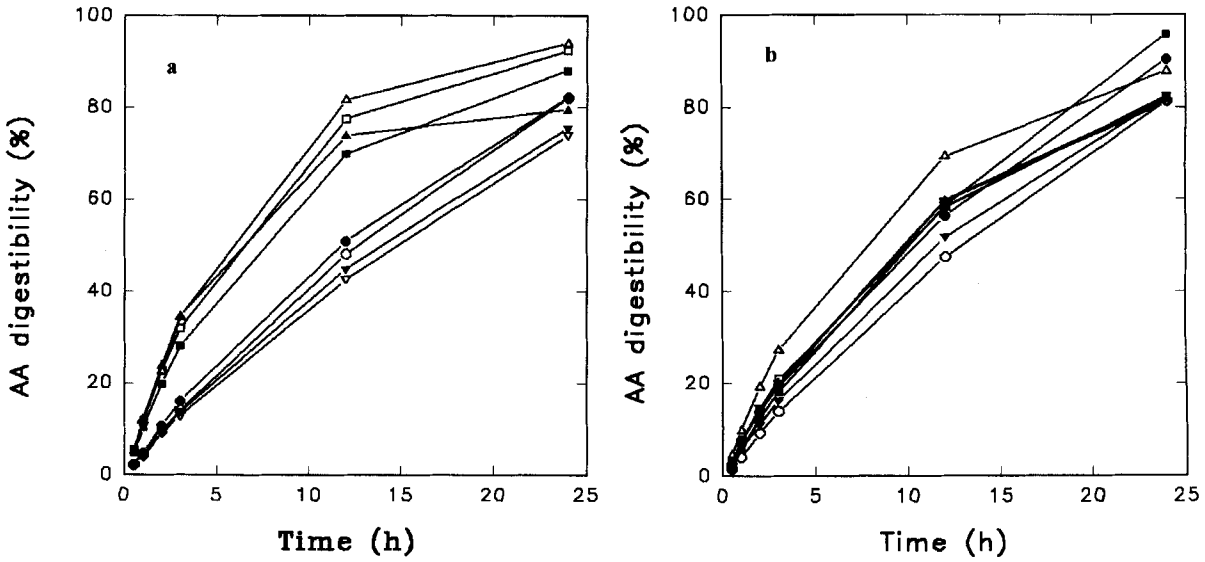

Figure 1. Kinetic curves of the release of amino acid residues durng the casein proteolysis in digestion cell according to C conditions (E_0/S_0, 1 : 50)
a: Asp (○), Thr (●), Ser (▽), Glu (▼), Tyr (□), Phe (■), Lys (△), Arg (▲)
b: Pro (○), Gly (●), Ala (▽), Val (▼), Met (□), Ile (■), Leu (△), His (▲)

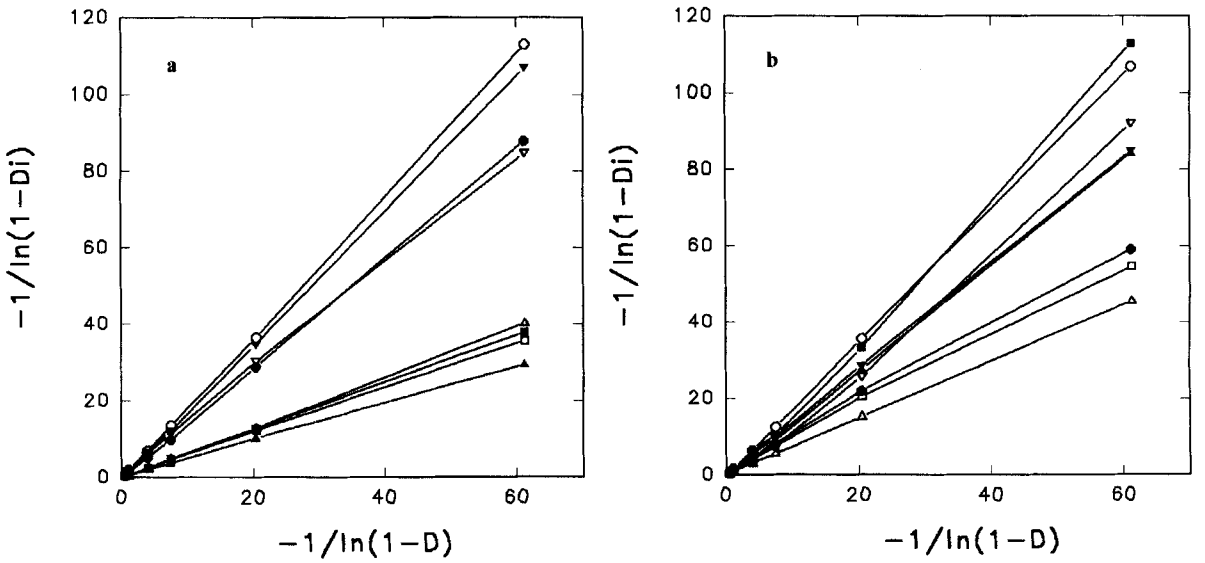

Figure 2. Proteolysis kinetic data from Fig. 1a, b are represented in new coordinates for calculation of rate constants k_i. Casein hydrolysed under C conditions. Dependences for different amino acid residues are denoted by analogy with Fig. 1

Table 1. Data set of experimentally obtained rate constants k_i

Protein	conditions*	Rate constants, k_i															
		Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
Casein	A	0.74	0.86	0.72	0.77	0.66	1.21	1.08	0.87	1.51	0.86	1.45	1.77	1.44	0.99	1.52	2.17
Casein	A	0.73	0.79	0.71	0.79	0.62	1.15	1.16	0.94	1.58	0.89	1.49	1.78	1.42	1.04	1.54	2.13
Casein	A	0.79	0.73	0.86	0.81	0.66	1.24	1.15	0.92	1.57	0.87	1.43	1.76	1.35	1.00	1.40	1.94
Casein	A	0.77	0.80	0.74	0.79	0.64	1.22	1.13	0.93	1.55	0.87	1.45	1.78	1.42	1.02	1.48	2.07
Casein	B	0.78	0.91	0.75	0.78	0.68	1.14	1.01	0.78	1.02	0.79	1.26	1.73	1.48	1.03	1.80	2.59
Casein	B	0.78	0.91	0.75	0.77	0.68	1.14	1.01	0.78	1.02	0.79	1.26	1.73	1.48	1.03	1.75	2.37
Casein	B	0.70	0.84	0.69	0.70	0.71	1.09	1.06	0.81	1.06	0.82	1.31	1.76	1.54	0.95	1.82	2.33
Casein	B	0.69	0.81	0.67	0.67	0.66	1.10	1.08	0.79	0.97	0.69	1.38	1.83	1.51	0.96	1.95	2.71
Casein	C	0.69	0.81	0.79	0.73	0.68	1.03	1.14	0.83	0.85	0.72	1.40	1.71	1.65	0.80	1.71	2.11
Casein	C	0.61	0.74	0.69	0.63	0.82	0.99	1.05	0.76	0.98	0.70	1.50	1.85	1.67	0.87	1.87	2.19
Casein	C	0.62	0.82	0.72	0.64	0.64	1.01	1.15	0.81	0.91	0.67	1.54	1.87	1.73	0.88	1.80	2.43
Rapeseed	A	1.00	0.93	0.95	0.75	0.53	0.96	1.15	1.02	1.52	1.10	1.16	2.51	1.70	1.01	0.89	1.17
Rapeseed	A	0.99	0.87	1.01	0.80	0.49	0.90	1.18	1.03	1.80	1.09	1.19	2.50	1.70	1.03	0.85	1.14
Rapeseed	A	1.05	0.95	0.96	0.87	0.61	0.96	1.16	0.96	1.30	1.01	1.12	1.59	1.54	0.92	0.94	1.21
Rapeseed	A	1.01	0.97	0.97	0.82	0.56	0.93	1.14	1.03	1.73	1.10	1.14	2.52	1.61	1.00	0.80	1.07
Rapeseed	B	1.07	1.07	0.92	0.72	0.69	0.90	1.09	0.89	1.10	1.09	1.26	2.25	1.84	0.92	0.91	1.07
Rapeseed	B	1.16	0.95	0.95	0.71	0.48	0.94	1.19	0.95	0.96	0.97	1.19	2.40	1.93	0.97	0.94	1.17
Rapeseed	B	1.07	1.07	1.05	0.65	0.65	0.86	1.08	1.07	0.89	1.09	1.29	2.24	1.97	1.09	0.87	1.07
Rapeseed	B	1.16	0.94	0.94	0.71	0.48	0.93	1.17	0.95	0.96	1.17	1.17	2.40	1.89	0.96	0.84	1.14
Rapeseed	C	1.08	0.83	1.03	0.61	0.55	0.86	1.25	0.88	0.92	1.00	1.43	2.47	2.44	0.92	0.78	1.12
Rapeseed	C	1.06	0.89	0.97	0.61	0.62	0.82	1.26	1.03	0.93	1.08	1.40	2.48	2.21	0.94	0.85	1.06
Rapeseed	C	1.05	0.93	0.93	0.61	0.52	0.89	1.20	0.94	1.00	1.13	1.39	2.47	2.17	0.91	0.92	1.17

* Different kinetic conditions A, B and C are described in Table 2

tive kinetics of amino acid residue release from the digestion cell (the slope was calculated by means of least-square method). The full set of rate constants k_i obtained as reciprocal values of slopes in all experiments is presented in Table 1. Mean rate constants for each of substrates, amino acids and proteolysis conditions are given in Table 2.

For casein (Table 2), whatever the conditions, the highest constants were obtained for Arg. On the other hand, it was impossible to select clearly the lowest constants among a group including Pro, Asp, Glu, Ser and Ile. For rapeseed (Table 2), whatever the conditions, the highest constants were obtained for Tyr, followed by Phe. The lowest constants were for Pro. Target residues for chymotrypsin and trypsin (Phe, Tyr, Arg and Lys) are released relatively fast being mostly on C-terminal of low molecular weight peptides or in the form of free amino acids. Other amino acid

Table 2. Rate konstants k_i for different conditions* and substrates

Amino acid	Casein			Rapeseed		
	A*	B	C	A	B	C
Asp	$0.76^{j,C**}$	$0.72^{hi,C}$	$0.64^{k,D}$	$1.01^{cdef,B}$	$1.12^{d,A}$	$1.06^{ef,AB}$
Thr	$0.79^{j,CD}$	$0.85^{g,BCD}$	$0.79^{ghij,D}$	$0.93^{def,AB}$	$1.01^{ef,A}$	$0.88^{gh,BC}$
Ser	$0.76^{j,B}$	$0.73^{hi,B}$	$0.73^{hijk,B}$	$0.97^{cdef,A}$	$0.96^{f,A}$	$0.98^{fg,A}$
Glu	$0.79^{j,A}$	$0.71^{hi,B}$	$0.66^{ijk,BC}$	$0.81^{jf,A}$	$0.70^{g,B}$	$0.61^{i,C}$
Pro	$0.64^{k,AB}$	$0.68^{i,A}$	$0.71^{ijk,A}$	$0.55^{g,B}$	$0.57^{h,B}$	$0.56^{i,B}$
Gly	$1.20^{f,A}$	$1.10^{e,B}$	$1.01^{ef,C}$	$0.94^{cdef,D}$	$0.91^{f,DE}$	$0.86^{h,E}$
Ala	$1.13^{g,B}$	$1.05^{e,C}$	$1.12^{e,B}$	$1.16^{c,B}$	$1.13^{cd,B}$	$1.24^{d,A}$
Val	$0.91^{i,B}$	$0.79^{gh,C}$	$0.80^{ghi,C}$	$1.01^{cdef,A}$	$0.97^{f,AB}$	$0.95^{gh,AB}$
Met	$1.55^{c,A}$	$1.02^{ef,B}$	$0.91^{fg,B}$	$1.59^{b,A}$	$0.97^{f,B}$	$0.95^{gh,B}$
Ile	$0.87^{i,B}$	$0.77^{ghi,C}$	$0.69^{ijk,C}$	$1.07^{cde,A}$	$1.08^{de,A}$	$1.07^{ef,A}$
Leu	$1.45^{de,A}$	$1.31^{d,B}$	$1.48^{d,A}$	$1.15^{cd,C}$	$1.22^{c,C}$	$1.41^{c,A}$
Tyr	$1.77^{b,B}$	$1.78^{b,B}$	$1.81^{b,B}$	$2.28^{a,A}$	$2.32^{a,A}$	$2.47^{a,A}$
Phe	$1.41^{e,D}$	$1.51^{c,D}$	$1.68^{c,C}$	$1.64^{b,C}$	$1.90^{b,B}$	$2.27^{b,A}$
His	$1.01^{h,A}$	$0.94^{f,A}$	$0.85^{gh,B}$	$0.99^{cdef,A}$	$0.98^{ef,A}$	$0.92^{gh,AB}$
Lys	$1.49^{d,B}$	$1.83^{b,A}$	$1.79^{bc,A}$	$0.87^{ef,C}$	$0.91^{f,C}$	$0.85^{h,C}$
Arg	$2.07^{a,B}$	$2.50^{a,A}$	$2.24^{a,B}$	$1.15^{cd,C}$	$1.11^{d,C}$	$1.12^{e,C}$

* Proteolysis conditions: A: E_0/S_0, 1:25; times: 3, 6, 9, 12, 15 h
B: E_0/S_0, 1:25; times: 1, 2, 3, 6, 24 h
C: E_0/S_0, 1:50; times: 0.5, 1, 2, 3, 12, 24 h

** Means with different small letters are significantly different within each column; means with different capital letters are significantly different within each row

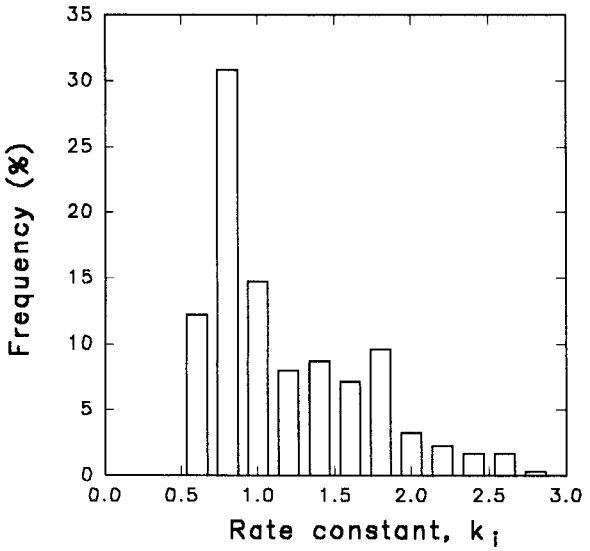

Figure 3. Constant distribution for casein (number of observed constants was 176 for all conditions)

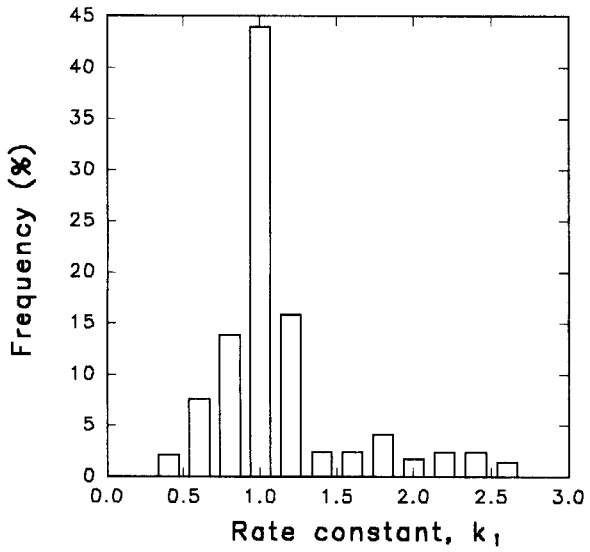

Figure 4. Constant distribution for rapeseed (number of observed constants was 176 for all conditions)

residues, which are considered as non-target for pancreatin enzymes, may be released relatively slow mostly as intrinsic residues in peptides.

The statistical comparison of constants (Fig. 3 and 4) showed that their distribution is broader for casein than for rapeseed proteins. Different experimental distributions of k_i indicate that the masking effect may change significantly the kinetic parameters. To elucidate relationship between masking and proteolysis kinetics in digestion cell, more detailed statistical analysis and computer modeling was decided to perform.

Coefficients of variation (CV) of the k_i, with the three highest CV's, are represented in Table 3 in comparison with the mean overall CV for the sixteen amino acids. It shows a quite adequate description of experimental data by the eq. (2) with mean error near 2%. The highest coefficients of variation were associated with amino acids such as Met or Arg; this could be related to losses during analysis [20]. For well determined Pro and His, the deviations could be attributed to the fact that the curves fitted the equation less well.

From the theoretical viewpoint, in experiments designed on the comparison of different substrates via the comparison of k_i parameters, E_0, S_0/E_0 and proteolysis time may be arbitrary, only a standard enzyme mixture is required [21], as well as standard physico-chemical conditions of enzyme action (as pH or T). Because these limitations were kept in this study, one can anticipate similar values for k_i in experiments with A, B and C conditions.

The invariability of rate constants under different experimental conditions was clearly determined for Ser and Tyr by DUNCAN's test (Table 2). The factor analysis was performed to clarify more precisely the influence of the experimental conditions. Constants for all amino acids were used as variables in the factor analysis. Scores are plotted for three factors (with variance 42%, 19% and 14% respectively) in Fig. 5 and Fig. 6. The plot of factor 1 vs. factor 2 allows one to identify separate and well-defined groups, each of them clearly related to one protein. The first two factors (Fig. 5), which represent the greater part of variance (61%), were not influenced by the conditions, contrary to factor 3 (14% of variance, Fig. 6). This factor was mostly determined by Met and Glu constants (this is deduced from the reference structure matrix not represented here). Fig. 6 clearly shows that B and C conditions were very close, or at least did not directly affect the constants, despite the different E_0/S_0 used (1 : 25 and 1 : 50 respectively).

The specific point qualifying the A conditions lies in the reduced time interval of the determination (the first point was 3 h for condition A). Even under the best conditions, amino acid determination implied systematic errors [22].

Table 3. Coefficients of variation (CV) of the rate constants k_i

A*				B				C			
Casein		Rapeseed		Casein		Rapeseed		Casein		Rapeseed	
Amino acid	CV	Amino acid	CV	Amino acid	CV	Amino acid	CV	Amino acid	CV	Amino acid	CV
Met	4.8[a]	Met	7.6[a]	Arg	4.3[a]	Pro	3.1[a]	His	2.7[a]	His	2.1[a]
Gly	4.5[a]	Arg	4.9[b]	His	3.0[ab]	His	2.5[a]	Met	2.6[a]	Arg	2.0[a]
Val	3.2[b]	Tyr	3.9[b]	Tyr	2.7[b]	Met	2.4[a]	Pro	2.0[a]	Val	1.8[a]
Mean**	2.1	Mean	2.9	Mean	2.2	Mean	1.5	Mean	1.3	Mean	1.1

* Proteolysis conditions A, B and C are described in Table 2
** Mean CV of 16 amino acids

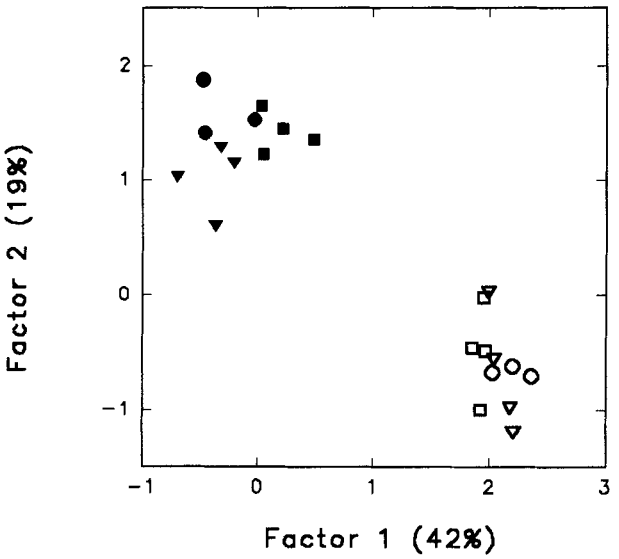

Figure 5. Score of factor 2 (19% of variance) vs. score of factor 1 (42% of variance). Casein hydrolysed under A conditions (■), B conditions (▼), C conditions (●). Rapeseed hydrolysed under A conditions (□), B conditions (▽), C conditions (○)

This, added to a reduced time interval in the calculation of constants, should increase the possibility of variation, especially if this is a critical point of kinetic measurements. It might take place for Met (this amino acid residue is important for factor 3), determination of which is complicated by the oxidation procedure.

Thus, B and C conditions are favourable from the metrological viewpoint. Meanwhile, in first approximation (within the 61% of variance), the assumption of independence of k_i from conditions is valid.

The data obtained in different sets of experiments were merged into one data set, which helps to characterize the digestibility of different substrates over all experimental conditions (Table 4). Except for Glu, Met (these constants

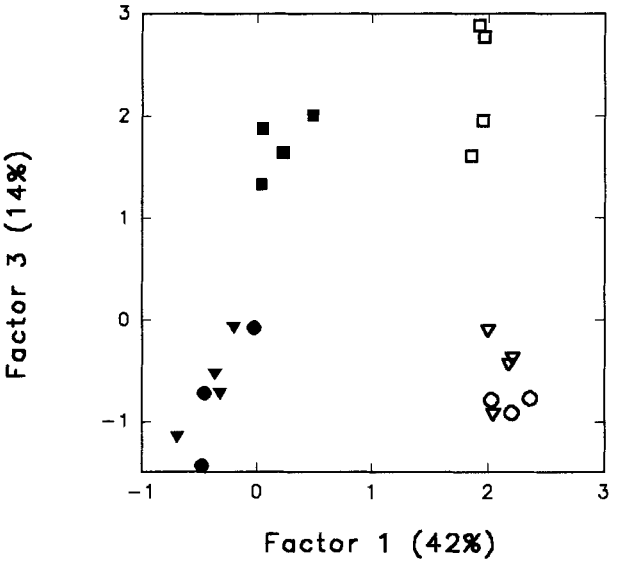

Figure 6. Score of factor 3 (14% of variance) vs. score of factor 1 (42% of variance). Casein hydrolysed under A conditions (■), B conditions (▼), C conditions (●). Rapeseed hydrolysed under A conditions (□), B conditions (▽), C conditions (○)

Table 4. Rate constants k_i obtained on the basis of the merged data set

Amino acid	Substrate		P
	Casein	Rapeseed	
Asp	0.71^{hi*}	1.06^{de}	0.01
Thr	0.81^{hg}	0.95^{ef}	0.01
Ser	0.74^{ghi}	0.97^{ef}	0.01
Glu	0.73^{ghi}	0.71^{g}	N.S.
Pro	0.68^{i}	0.56^{h}	0.01
Gly	1.11^{e}	0.90^{f}	0.01
Ala	1.10^{e}	1.17^{cd}	0.01
Val	0.84^{fg}	0.98^{ef}	0.01
Met	1.18^{e}	1.19^{cd}	N.S.
Ile	0.79^{ghi}	1.07^{de}	0.01
Leu	1.41^{d}	1.25^{c}	0.01
Tyr	1.79^{b}	2.35^{a}	0.01
Phe	1.52^{c}	1.91^{b}	0.01
His	0.94^{f}	0.97^{ef}	N.S.
Lys	1.69^{b}	0.88^{f}	0.01
Arg	2.28^{a}	1.13^{cd}	0.01
Target**	1.82	1.57	0.01
Non-target	0.92	0.98	0.01
Δ***	0.90	0.59	0.01

* Means with different superscripts within each column are significantly different. N.S. = Non significantly different within each row

** Target amino acids including Tyr, Phe, Lys and Arg

*** Difference between target and non-target constants

being dependent on proteolysis conditions) and His, other constants were all statistically different when compared according to substrate. The largest difference was noted for trypsin-dependent constants (k_i for Arg and Lys were twice large for casein as for rapeseed). On the contrary, chymotrypsin-dependent constants were higher for rapeseed than for casein (approximately 1.3 times). The group of slowly released amino acids (Asp, Thr, Ser and Ile) showed higher k_i values for rapeseed. However, Pro, which had the lowest k_i value, was released more slowly for rapeseed.

The difference between constants represented in Table 4 reflects the specificity of enzyme action with respect to target amino acid residues. But this effect is obscured by the fact that, besides free amino acids readily released by carboxypeptidases and amino acid residues on the C-terminal of peptides being released by endopeptidases, the amino acid residues inside peptide chains are released non-specifically. The constant for amino acid residue release as free amino acid k_i^{aa} must be closely connected to both endopeptidase and exopeptidase activities, because the non-specific release component was not involved in the k_i^{aa}constants. The data presented in Table 5 show greater differences in these constants for a given substrate than those of Table 4. The most significant differences between substrates were for k_i^{aa} constants of Arg and Lys (two times greater for casein); Tyr, Phe and Pro (greater for rapeseed). The ratio of maximum to minimum k_i^{aa} presented in Table 5 ($k_{Arg}^{aa}/k_{Pro}^{aa}$ for casein and $k_{Tyr}^{aa}/k_{Pro}^{aa}$ for rapeseed) is at least 10.

The results of computer modeling performed by means of PROTEOLYSIS program package are presented in Fig. 7 as dependences of AAD vs. degree of proteolysis $d = N/(B_d + B_m + N)$. Computer simulation implies the simplification of process, therefore, only chymotrypsin was taken

Table 5. Rate constants for free amino acid release (k_i^{aa})

Amino acid	Substrate		P
	Casein	Rapeseed	
Pro	0.01^{h*}	0.19^{h}	0.01
Gly	0.28^{g}	0.39^{fgh}	N.S.
Ala	0.48^{ef}	0.60^{efg}	N.S.
Val	0.35^{fg}	8.59^{efg}	0.01
Met	0.96^{d}	1.05^{c}	N.S.
Ile	0.38^{fg}	0.72^{de}	0.01
Leu	1.03^{cd}	0.87^{cd}	0.01
Tyr	1.29^{bc}	1.92^{a}	0.01
Phe	1.11^{cd}	1.40^{b}	0.01
His	0.61^{e}	0.623^{ef}	N.S.
Lys	1.15^{bc}	0.56^{efg}	0.01
Arg	1.88^{a}	0.89^{cd}	0.01
Target**	1.36	1.19	0.01
Non-target	0.51	0.63	0.01
Δ***	0.85	0.56	0.01

* Means with different superscripts within each column are significantly different
** Target amino acids including Tyr, Phe, Lys and Arg
*** Difference between target and non-target constants

into account. Proteolysis by chymotrypsin was simulated for two substrates with different values of demasking parameter k_d/k_h and the same sequence of amino acid residues. In case of demasked peptide chain ($k_d/k_h = 4$), the specific amino acid residues Tyr and Phe are liberated more rapidly than it goes for masked substrate with $k_d/k_h = 0.04$. Contrary, the non-target amino acid residues (Gly and Val, for example) are liberated more slowly for demasked substrate than for masked one. Thus, kinetic curves are spread broader if there is no limitation connected with masking (Fig. 8). These kinetic curves are distributed in the sector marked with vertical shading for demasked substrate and in the more narrow sector for masked substrate (marked with grating). Kinetic parameters used in PROTEOLYSIS program for α-chymotrypsin specificity (first approach for specificity constant evaluation from [11]) are defined more precisely than that for other proteolytic enzymes, meanwhile the regularity reported is also valid for an arbitrary enzyme or enzyme mixture.

Theoretical analysis and computer modeling show that the greater the extent of masking the smaller must be the range of variation among the rate constants. Under additional masking, the hydrolysis of target but masked bonds is reduced. On the contrary, less specific but demasked bonds are more susceptible to hydrolysis. Thus under additional masking, the distribution of k_i must be narrower. Comparison of the experimentally determined distributions for masked rapeseed and demasked casein confirms this peculiarity (Fig. 3 and Fig. 4).

Because of experimental complexity of distribution determination for all rate constants, one needs a simpler criterion for estimation of state of masking. As a measure of masking effects one can use difference Δ in the rate constants for target (Tyr, Phe, Lys and Arg) and non-target amino acid residues (other residues). This parameter is greater for casein (0.90) than for rapeseed (0.59) (Table 4). Release kinetics for free amino acids reveals the same peculiarity: viz., Δ is greater for casein (0.85) comparing with rapeseed (0.56) (Table 5).

One can anticipate that the difference between target and non-target rate constants can reflect the difference in masking of other protein substrates [19, 23] which are able partially to retain their tertiary structure after peptic predigestion. The illustration is shown in Fig. 9, where the native β-lactoglobulin [23] and rapeseed proteins represent substrates with greater masking (Δ = 0.53 and 0.59) than casein (Δ = 0.90). This high level of masking for β-lactoglobulin is probably provided by tertiary structure [14], while for rapeseed protein concentrates the most important factors seem to be the incomplete solubility and the presence of glucosinolates, phytic acid and phenolic compounds which

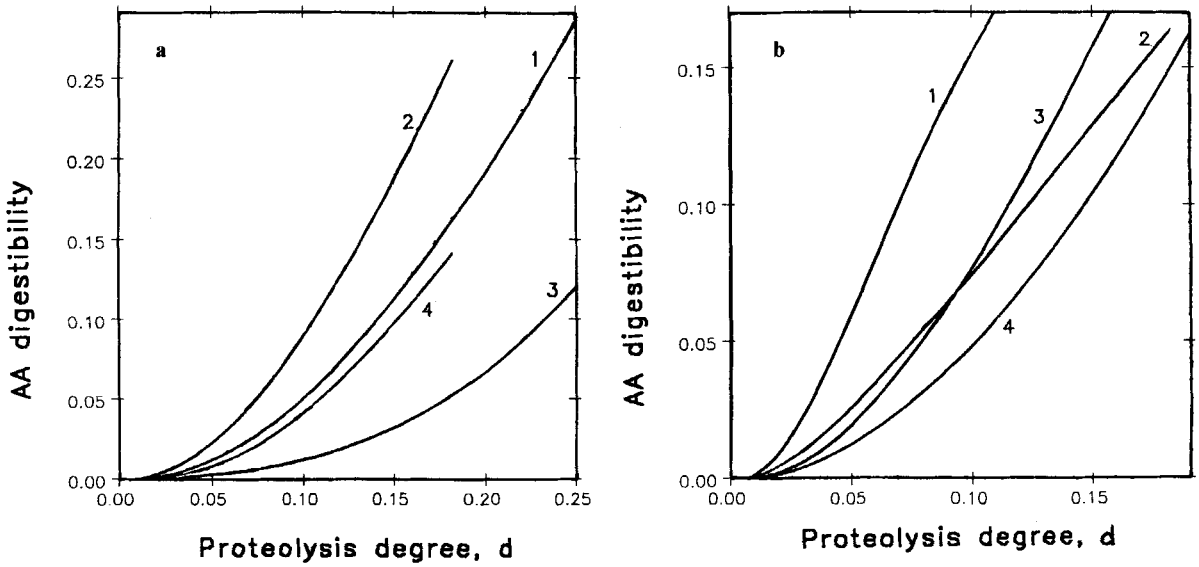

Figure 7. Computer simulation of proteolysis kinetics by means of PROTEOLYSIS program. AAD was calculated for proteolysis products with length less than 6 residues. Sequence of amino acid residues for β-casein and specificity parameters for α-chymotrypsin from [11] were used for simulation
a: Specific residues Tyr and Phe; Tyr, $k_d/k_h = 0.04$(1); Tyr, $k_d/k_h = 4$(2); Phe, $k_d/k_h = 0.04$(3); Phe, $k_d/k_h = 4$(4)
b: Non-specific residues Val and Gly; Val, $k_d/k_h = 0.04$(1); Val, $k_d/k_h = 4$(2); Gly, $k_d/k_h = 0.04$(3); Gly, $k_d/k_h = 4$(4)

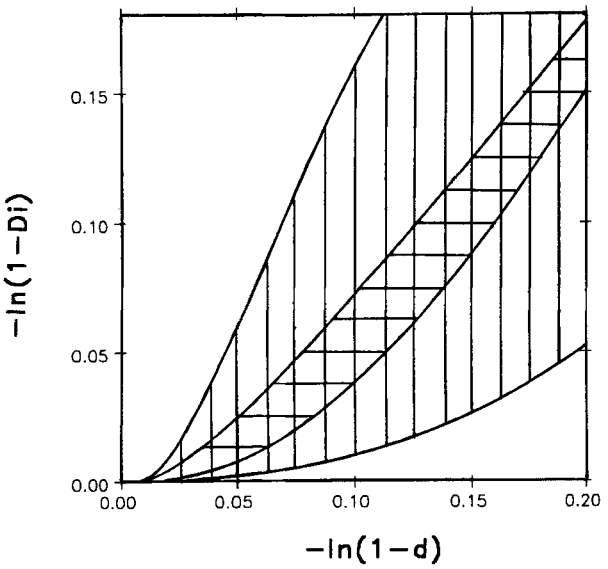

Figure 8. Computer stimulation of proteolysis kinetics. Results are presented in logarithmic coordinates. Masked substrate with $k_d/k_h = 0.04$ (▦); demasked substrate with $k_d/k_h = 4$ (▥)

bind with rapeseed proteins and reduce their nutritive value [24, 25]. Milk whey concentrate containing partially denaturated whey proteins gives greater $\Delta = 0.85$ comparing with native β-lactoglobulin ($\Delta = 0.53$). Demasking of whey proteins in preparation used was additionally increased by heating which led to increase in Δ from 0.85 to 1.01.

Thus, the method of determination of rate constants proposed in the present work enables quantitative comparison between digestion kinetics at the pancreatic stage of digestion for substrates with different masking. The various kinetics of release of each amino acid residue can be described by the same simple equation (eq. 2), and then the relative rate constants can be compared.

In contrast to other methods where nitrogen digestibility or total amino acids from digestion products were analysed, the proteolysis in dialysis reactor allows to study the kinetics for amino acids and low molecular weight peptides which are the end products of the lamina digestion. Our study shows that this fractions of whole hydrolysate are considerably affected by the state of masking of protein substrate. In kinetic aspect, most noticeable are the changes in distribution of rate constants and in difference between target and non-target rate constants. This approach could later be tested with other protein substrates of various origins and nutritive values.

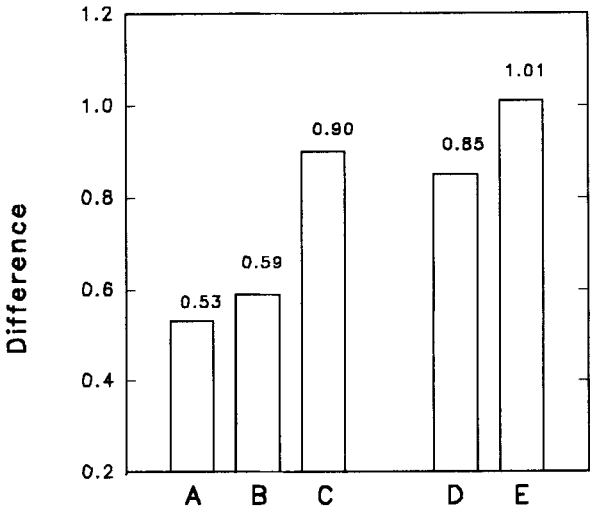

Figure 9. Difference between target and non-target constants (Δ) for various protein substrates: native β-lactoglobulin, A; rapeseed, B; casein, C; milk whey concentrate, D; heated milk whey concentrate, E

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