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Quantification of Chymosin Action on Nonlabeled κ-Casein-Related Peptide Substrates by Ultraviolet Spectrophotometry: Description of Kinetics by the Analysis of Progress Curves

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A method is described for quantifying the proteolytic action of the milk-clotting enzyme chymosin on small and medium-sized peptide substrates by monitoring the decrease of absorbance at 230 nm during cleavage. The method is illustrated by the determination of the kinetic parameters of the specific splitting of a κ-casein-related hexa- and pentadecapeptide by chymosin. The results are in good agreement with those found earlier with the same enzyme/substrate system by using an automated ninhydrin method. Erroneous results were obtained when the kinetic data were derived from one single progress curve. The significance of initial rate measurements for calculating correct kinetic parameters is briefly discussed. The usefulness of single progress curves measured at different initial substrate concentrations for obtaining information about the mechanism of the enzymic reaction is demonstrated.

KEY WORDS: proteolysis; enzyme assay; chymosin; enzyme kinetics; peptide substrates; uv spectrophotometry.

Chymosin (rennin; EC 3.4.23.4) forms the main enzymatic component of calf rennet used as milk coagulant in the process of cheese making. It is not surprising therefore that the activity of the enzyme has long since been expressed as milk clotting units. A disadvantage of taking the clotting strength of the coagulant as a measure of enzyme activity is the possible variability of the composition of the substrate, i.e., milk. To meet this problem as much as possible, clotting strength is mostly established by comparison with a reference enzyme preparation, the activity of which has been carefully determined using a well-defined substrate (1). The clotting of milk is initiated by the specific proteolytic cleavage of κ-casein by chymosin (or any other coagulating enzyme) which then brings about a destabilization of the colloidally dispersed casein micelles.

The synthesis of peptide substrates for chymosin (2–5) has made possible the determination of the proteolytic activity of the milk-clotting enzyme in a direct way. Hexapeptide esters having amino acid sequences closely related to the region around the chymosin-sensitive Phe(105)–Met(106) linkage of bovine κ-casein have appeared well suited for this purpose (6,7). Measurement of the activity could be achieved either by labeling the synthetic peptide through the introduction of a Phe(NO_2) group and following the cleavage reaction at 310 nm (6,8) or by monitoring the splitting of the unlabeled peptide by an automated ninhydrin method (9–11). Using the latter assay method the kinetics of chymosin action on a series of κ-casein-related peptides have been determined to study the interaction between the enzyme and its substrate (12–15). As the ninhydrin reaction is not the most direct assay method and may also be subject to interference by other ninhydrin-positive substances, we became interested in finding an alternative procedure to quantify the chymosin action on nonlabeled peptide substrates.

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The commercial introduction of modern spectrophotometers which can be interfaced to a desk top computer, has provided a means to obtain spectra with excellent signal-to-noise characteristics. The use of such equipment allows the accurate measurement of small changes in optical density, which enables the reliable evaluation of progress curves for the determination of the kinetic constants of enzymatic reactions.

The present paper describes a spectrophotometric method to measure directly the proteolytic action of chymosin on peptide substrates at 230 nm. The feasibility of the method for peptide substrates having up to 15 amino acid residues is illustrated by the determination of the kinetic parameters of the specific cleavage of a κ-casein-related hexa- and pentadecapeptide by chymosin. The results are compared with those obtained in a previous study by the ninhydrin method.

The dangers encountered with calculating the results from one single progress curve instead of from a series of initial rate determinations are also discussed.

MATERIALS AND METHODS

The hexapeptide ester Leu–Ser–Phe–Nle[1]–Ala–IleOMe was prepared according to Schattenkerk (16) and the pentadecapeptide His–Pro–His–Pro–His–Leu–Ser–Phe–Met–Ala–Ile–Pro–Pro–Lys–LysOH as described by Visser *et al.* (14). These peptides are specifically split by chymosin at the Phe–Nle and Phe–Met position, respectively. The hexapeptide was dissolved at a concentration of 2 mM in 0.05 M sodium acetate buffer (pH 4.7) by sonication; for kinetic measurements this stock solution was diluted (concentration range 0.2–2.0 mM) with the same buffer. The pentadecapeptide was dissolved either in the above acetate buffer (pH 4.7) or in a potassium phosphate buffer (pH 6.6; I = 0.05 M); for kinetic measurements stock solutions (0.15 mM) were diluted with one of the above buffers (final peptide concentration 0.02–0.15 mM). Buffers and substrate stock solutions were filtered through a 0.22-μm filter (Millex-GV, Millipore) before use. The final concentrations of the substrate in the stock solutions were established by amino acid analysis.

Chymosin was purified by DEAE-Sephadex chromatography according to De Koning (17); its milk clotting activity amounted to 6.5×10^6 Soxhlet units (1,18). An appropriate amount (4–10 μl) of enzyme solution in 0.05 M sodium acetate buffer containing 1 M sodium chloride (pH 5.2) was added to 500 μl of the diluted substrate stock solution which had been preequilibrated at 30°C in a quartz cuvette of 1.0-cm optical path length. Final enzyme concentrations were 82 and 7.9 nM in the reactions with the hexapeptide and pentadecapeptide, respectively (enzyme solutions of different concentrations were used for the addition to the two peptide substrates). Concentrations were established on a weight basis assuming a molecular weight of 30,000 (1) for the enzyme.

Measurement of the absorbance change at 230 nm as a function of time was started within 30 s after mixing of enzyme and substrate solution. Use was made of a Cary 219 spectrophotometer interfaced to an HP-86 computer (Hewlett–Packard) via a GPIO-interface on the HP-86 connected to the digital interface port (DIP) of the Cary 219. The relatively high absorbance of the unreacted substrate was suppressed using the rear beam attenuator accessory of the spectrophotometer. The computer was used both for data collection and analysis of the progress curves. The time course of the absorbance was followed by taking approximately every 3 s an absorbance measurement which was the average of 10 absorbance readings done within 1 s (sampling rate, 12 s^{-1}). For one progress curve 200–500 data points were collected. The derivative of the absorbance vs. time curve was obtained using a polynomial fitting procedure. The calculation of the reaction rate at a particular point of the curve was performed by fitting a fourth order polynomial to 41 data points ar-

[1] Abbreviation used: Nle, norleucine.

ranged symmetrically around the point of interest. Reaction rates and substrate concentrations were used to construct Hanes (s/v vs. s) plots (19) for the various progress curves.

RESULTS AND DISCUSSION

As seen in Fig. 1, the change in molar absorptivity ($\Delta\epsilon$) due to splitting of the hexapeptide ester by chymosin shows an optimum near 227 nm. The absorbance of the intact peptide increases almost exponentially around this wavelength. We found the measurement at 230 nm a reasonable compromise for proper handling of substrate concentrations up to 2 and 0.2 mM of the hexa- and pentadecapeptide, respectively, in the buffer systems used. At these concentrations the relatively high initial absorbances (up to about 2 absorbance units if no correction had been made for cuvette and buffer blanks) could be easily compensated for by the rear beam attenuator accessory of the spectrophotometer; as a result the starting readings for the intact hexa- and pentadecapeptide were in the regions of 0.5–0.8 and 0.02–0.14 absorbance unit, respectively. Under these conditions we found for both peptides an excellent linear relationship

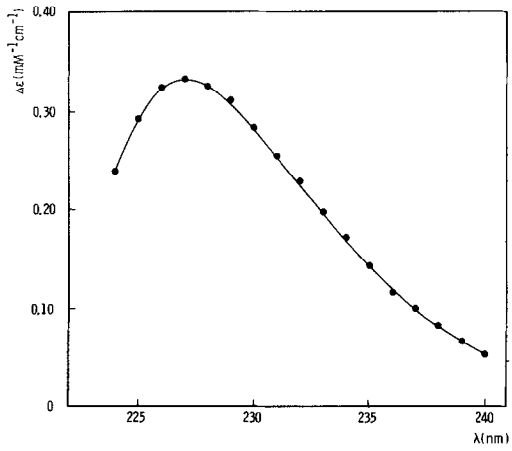

FIG. 1. Change of the molar absorptivity $\Delta\epsilon$ as a function of the wavelength, as measured for the specific splitting of the hexapeptide Leu-Ser-Phe-Nle-Ala-IleOMe by chymosin in 0.05 M sodium acetate, pH 4.7, at 30°C.

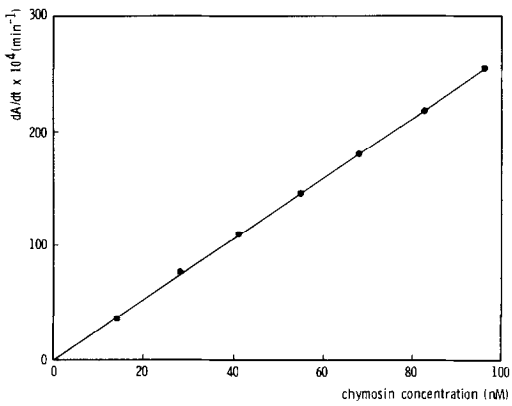

FIG. 2. Relationship between the initial rate of cleavage of the hexapeptide (1 mM) measured at 230 nm and the concentration of the chymosin preparation used. Conditions as in Fig. 1.

between absorbance and concentration (results not shown). At 230 nm $\Delta\epsilon$ for the hexapeptide hydrolysis measured in 0.05 M sodium acetate (pH 4.7) was 0.282 ± 0.007 mM^{-1} cm^{-1} ($n = 14$).[2] For splitting of the pentadecapeptide in the same buffer $\Delta\epsilon$ was 0.396 ± 0.007 mM^{-1} cm^{-1} ($n = 27$), whereas in the potassium phosphate buffer (pH 6.6) a value of 0.308 ± 0.018 mM^{-1} cm^{-1} ($n = 42$) was found with this substrate.

In Fig. 2 the proportionality of initial velocity to enzyme concentration for the cleavage of the hexapeptide by chymosin at pH 4.7 is shown. Taking the enzyme concentration within this range, the kinetics of this cleavage reaction were determined. Figure 3 represents a typical progress curve for the lowest substrate concentration used. The V_{max} and K_m values were determined from a Hanes plot (Fig. 4a, solid line) in which s/v values had been obtained by extrapolating the Hanes plots of individual progress curves to the initial substrate concentrations. Although the individual progress curves yield linear Hanes plots (cf. Fig. 4a, dashed lines), these plots do not coincide when different initial substrate concentrations are considered. K_m values obtained from those

[2] Standard error of the mean obtained from n determinations.

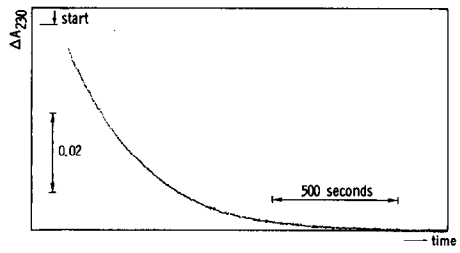

FIG. 3. Progress curve for the cleavage of the hexapeptide (0.19 mM) by chymosin. Measurement was started about 30 s after mixing of the substrate and enzyme solutions. Conditions as in Fig. 1. Enzyme concentration 82 nM.

individual progress curves vary considerably with the initial substrate concentration, while V_{max} remains approximately constant. This behavior can be described by assuming that competitive product inhibition occurs. When the ratio of the apparent K_m and V_{max} from the individual progress curves is plotted as a function of the initial substrate concentration, a linear dependence is observed and a value for the inhibition constant of 0.84 mM is obtained (Fig. 4b). It should be mentioned that the anomalous behavior observed when calculating the kinetics from a single progress curve could also be explained by inactivation of the enzyme during the assay. However, in our case the latter possibility was ruled out by experiments in which an additional (fixed) amount of substrate was added at different times (up to 40 min) after completion of the reaction. The results of these experiments showed no detectable enzyme inactivation within the time period of measurement.

Figure 5 represents a typical progress curve for the cleavage of the pentadecapeptide at the lowest concentration used (pH 4.7). In Fig. 6, Hanes plots derived from a series of initial rate experiments (solid lines) and from two individual progress curves (dashed lines) are shown. In these cases no clear indications for competitive product inhibition were observed: $(K_m/V_{max})_{app}$ vs. s patterns do not suggest a linear relationship (results not shown). This would mean that in these cases K_I is much larger than K_m.

The kinetic parameters, k_{cat}, K_m, and k_{cat}/K_m for the action of chymosin on the hexa- and pentadecapeptide are listed in Table 1. It is seen that the present results are in good agreement with those obtained earlier (13–15) by using an automated ninhydrin method (11).

The method described here can be used to monitor the (limited) proteolysis of short-chain peptide substrates; it seems that under our experimental conditions the size of the 15-residue peptide is almost the limit for accurate measurement of splitting rates.

It should be noted that the danger exists (as

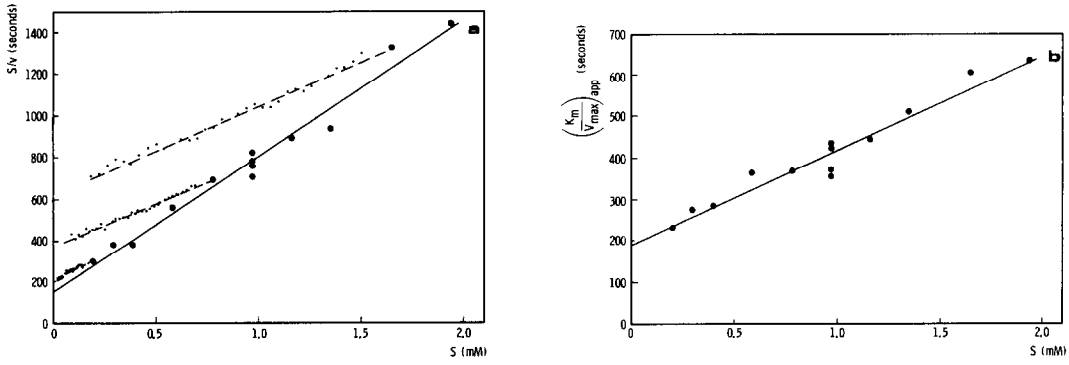

FIG. 4. (a) Hanes plots for the cleavage of the hexapeptide by chymosin. Dashed lines through small dots, derived from individual progress curve; solid line through heavy dots, from values at initial substrate concentrations obtained by extrapolating Hanes plots of individual progress curves. (b) Relationship between $(K_m/V_{max})_{app}$ and the substrate concentration for the cleavage of the hexapeptide by chymosin. Enzyme concentration 82 nM; further conditions as in Fig. 1.

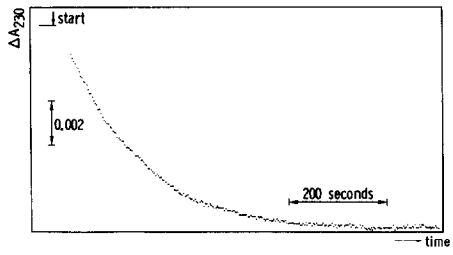

FIG. 5. Progress curve for the cleavage of the pentadecapeptide (0.024 mM) by chymosin at pH 4.7 and 30°C. Measurement was started about 30 s after mixing of the substrate and enzyme solutions. Enzyme concentration 7.9 nM.

shown by the results obtained with the hexapeptide) of getting erroneous results when kinetic parameters are determined from a single progress curve. On the other hand, an important advantage of using individual progress curves instead of or in addition to carrying out initial slope experiments, is the fact that more information about the mechanism of the reaction (in particular the possible occurrence of product inhibition) may be obtained. The extrapolation method used in the present study (cf. Fig. 4a and Fig. 6) is based on the same idea as the one advocated many years ago by Niemann and his co-workers (20,21). As also pointed out by Cornish-Bowden (22) this method should in principle be preferred to the measurement of initial slopes. It can be calculated that by using the latter procedure K_m and V_{max} values obtained are systematically a little too high. This might explain the slight differences between the results of the ninhydrin method and those of the present spectrophotometric procedure (see Table 1).

For the determination of chymosin activity the hexapeptide Leu – Ser – Phe – Nle – Ala–IleOMe as well as its analog Leu–Ser–Phe(NO_2)–Nle–Ala–LeuOMe have been used as reference substrate (6,7,23). The splitting of the latter substrate containing the chymosin-sensitive Phe(NO_2)–Nle linkage can be followed spectrophotometrically at 310 nm. Up till now the more laborious ninhydrin method was used to monitor the cleavage of the Phe–Nle bond in the nonlabeled substrate. Using the latter rather than the NO_2-labeled hexapeptide is advantageous, not only from a synthetical point of view but also because one of the splitting products of the labeled substrate (i.e., Leu–Ser–Phe(NO_2)OH) has been reported to be somewhat unstable under uv-light exposure (24). Furthermore, the Phe(NO_2)-containing hexapeptide shows in its cleavage by chymosin substrate inhibition at concentrations exceeding 1 mM (13). A point in favor of using the NO_2-labeled hexapeptide might be the higher $\Delta\epsilon$ value found for the splitting reaction at pH 4.7 monitored at 310 nm, but under our conditions the lower $\Delta\epsilon$ value did

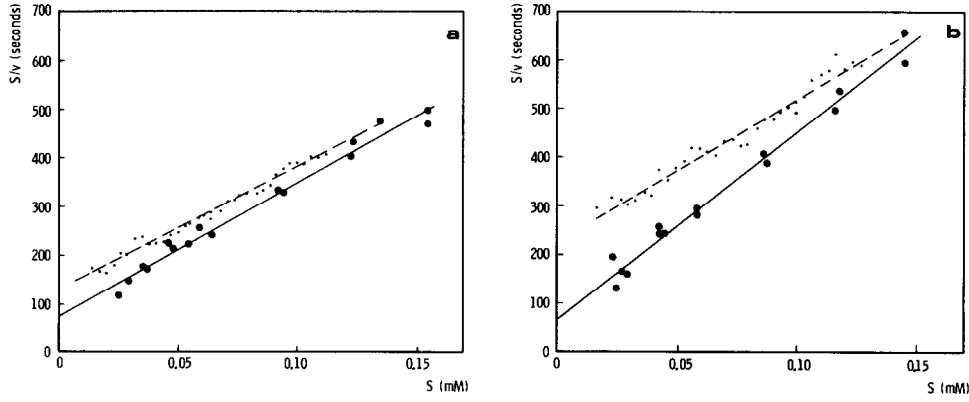

FIG. 6. Hanes plots for the cleavage of the pentadecapeptide by chymosin at 30°C and at pH 4.7 (a) or pH 6.6 (b). Dashed and solid lines as in Fig. 4. Enzyme concentration 7.9 nM.

TABLE 1

KINETIC PARAMETERS FOR THE CLEAVAGE OF THE κ-CASEIN-RELATED HEXA- AND PENTADECAPEPTIDE AS DETERMINED BY SPECTROPHOTOMETRY AT 230 nm: COMPARISON WITH RESULTS OBTAINED WITH THE AUTOMATED NINHYDRIN METHOD DESCRIBED IN (11)[a]

		Present method[b]			Ninhydrin method[c]		
Substrate	pH	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
κ(103-[Nle106]-108)OMe[d]	4.7	18.6	0.24	77.5	24.9	0.36	69.2
	4.7	46.1	0.026	1773	61.9	0.028	2208
		53.4	0.039	1368			
κ(98–112)OH[e]							
	6.6	38.4	0.021	1830	48.2	0.023	2123
		32.7	0.018	1847			

[a] Kinetic parameters derived from initial rate determinations at different substrate concentrations using either the extrapolation (present method) or the direct initial slope procedure (ninhydrin method). For further experimental conditions see the text.

[b] Pentadecapeptide results are given of duplicate experiments done at a time interval of several weeks.

[c] Data taken from Refs. (13–15).

[d] Leu–Ser–Phe–Nle–Ala–Ile methyl ester.

[e] His–Pro–His–Pro–His–Leu–Ser–Phe–Met–Ala–Ile–Pro–Pro–Lys–Lys.

not preclude a rapid and accurate measurement.

The present method of direct monitoring and quantitation of the proteolysis at 230 nm has been successfully applied in the determination of relative amounts of chymosin and pepsin in bovine and calf rennets using the above-mentioned nonlabeled hexapeptide as the reference substrate (results to be published). Using this method the proteolytic activity of the chymosin preparation applied in the present and previous (12–15) studies was established as 540 ± 30 mkat/kg under specified conditions (i.e., measuring the cleavage of 1.05 mM hexapeptide ester in 0.05 M acetate buffer, pH 4.7, at 30°C). The procedure described in this paper should also be suitable for the quantification of proteolysis in other enzyme/substrate systems provided that the experimental conditions, such as size and concentration of the substrate, do not deviate too much from those applied in the present study.

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