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Kinetics of Action of Chymosin (Rennin) on Some Peptide Bonds of Bovine β-Casein†

Christophe Carles* and Bruno Ribadeau-Dumas

ABSTRACT: The first steps of proteolysis of bovine β-casein by chymosin were studied quantitatively by using reverse-phase high-performance liquid chromatography (RP-HPLC). Although chymosin has a broad specificity, it has been possible to selectively study the hydrolysis of two bonds (Ala-189–Phe-190 and Leu-192–Tyr-193) by choosing appropriate conditions. The disappearance of the substrate and the appearance of the reaction products as a function of time were followed at 220 nm by RP-HPLC. For concentrations where β-casein was in a micellar form, the Michaelian parameters corresponding to the cleavage of bond 192–193 were determined by measuring initial rates of reaction at different substrate concentrations in a time period for which splitting of bond 189–190 was negligible. The following results were obtained: $k^1_{cat} = 1.54\ s^{-1}$, $K^1_m = 0.075$ mM, and $k^1_{cat}/K^1_m = 20.6\ mM^{-1}\ s^{-1}$. Under conditions where the protein was in a monomeric state, the following parameters were determined for the splitting of bond 192–193 by integrating the Michaelis equation: $k^2_{cat} = 0.056\ s^{-1}$, $K^2_m = 0.007$ mM, and $k^2_{cat}/K^2_m = 79.7\ mM^{-1}\ s^{-1}$. Under the latter conditions the four enzymic reactions involved in the cleavage of bonds 189–190 and 192–193 were first-order reactions. The four corresponding apparent rate constants were calculated by using a computer program. Excellent agreement was obtained between concentrations of four molecular species measured during the reaction period and those calculated by using the four apparent rate constants.

Chymosin, formerly named rennin (EC 3.4.23.4), is an aspartyl proteinase of broad specificity secreted in the stomach of some newborn animals (ruminants and a few other species). Its natural protein substrates are the caseins (α_{s1}, α_{s2}, β, and κ) that exist in cow milk, together with inorganic ions, as high molecular weight coaggregates, the micelles. The first event that follows milk ingestion in the calf is its coagulation in the stomach. It is due to the splitting by chymosin of a single bond, Phe-105–Met-106 (Delfour et al., 1965), in κ-casein. This destroys the micelle-stabilizing properties of this protein and leads to micelle aggregation through hydrophobic interactions. Chymosin contributes to the digestion of the coagulum by a slower action on a number of peptide bonds of the four caseins. However, it has been known for a long time, from in vitro studies, that a few bonds in α_{s1}- and β-casein are highly susceptible to chymosin.

Using gel electrophoresis, Creamer et al. (1971) observed the appearance of three bands (βI, βII, and βIII, in their order of appearance) when β-casein (209 residues) was incubated

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with chymosin. Band βI was shown by Visser & Slangen (1977) to correspond to a mixture of peptides 1–189 and 1–192. These authors observed that bond Leu-192–Tyr-193 was more susceptible to chymosin than bond Ala-189–Phe-190. From their digests they also isolated peptides 190–192, 190–209, and 193–209. Later on, Visser (1981) indicated that bond Leu-3–Glu-4 was also chymosin sensitive. As peptide 1–3 (Arg-Glu-Leu) is neutral under the conditions of gel electrophoresis, the occurrence of peptides 4–189 and 4–192 in band βI, as well as peptide 4–209 in band β-casein, cannot be excluded.

In the present work, we have studied by reverse-phase high-performance liquid chromatography (RP-HPLC) the primary action of chymosin on bovine β-casein (giving only band βI). To our knowledge, the present study is the first one for which HPLC has been used to study enzyme kinetics involving a proteinase acting on a protein substrate.

Materials and Methods

Chemicals and Enzymes. Lichrosolv organic solvents from Merck (Darmstadt, FRG) were used for HPLC. All other chemicals (analytical grade) were from the same firm. Buffers and solvents for HPLC were filtered through Millipore 0.45-μm filters (Millipore Corp., Bedford, MA) and degassed under vacuum before use. Carboxypeptidase A (CPA, 35 units/mg) was from Boehringer (Mannheim, FRG).

The proportion of active enzyme in the freeze-dried chymosin preparation obtained from Sigma (St Louis, MO) was determined as described by Martin et al. (1981). It was 19.3% (w/w). The preparation was shown to be free of pepsin by using the immunological technique reported by Collin et al. (1982).

Substrate Preparation. β-Casein A[1] was prepared as described by Mercier et al. (1968) from the milk of a cow homozygous at the four casein loci. The concentration of the casein solutions was determined, after filtration on 0.45-μm filters, from the extinction coefficient $E_{280nm}^{1mg/mL} = 0.46$ given by Swaisgood (1982).

Enzymic Reactions. β-Casein was digested with chymosin at 30 °C in 50 mM sodium citrate buffer, pH 6.2, and NaN_3 (0.1% w/v). Aliquots were taken at intervals, and the reaction was stopped by adding a known volume of concentrated ammonia to bring the pH to 9–10. It was shown that inhibition was immediate and irreversible. Volumes of 10–30 μL were injected on the HPLC column after filtration on 0.45-μm filters. However, in the experiments performed with low substrate concentration, 500-μL injections were necessary. As in these cases the high pH and volume prevented suitable fractionation, the reactions were carried out in the presence of an internal standard (dansylserine). The aliquots were freeze-dried after ammonia addition, taken in a small volume of water, and filtered. This made possible injections of 50 μL of nearly neutral solutions.

Some peptides were treated with CPA in order to examine their C-terminal end. Dry peptides were dissolved in 0.2 M *N*-ethylmorpholine brought to pH 8.6 with acetic acid. Phenylmethanesulfonyl fluoride (PMSF) was added (1 mM final concentration) to prevent the action of serine proteinases that contaminated the CPA preparation. The reactions were performed at 37 °C for 12 h with an enzyme/substrate (E/S) ratio of 1/70 (w/w). The digests were freeze-dried and submitted to amino acid analysis.

Amino Acid Analyses. They were carried out on a LC 5000 amino acid analyzer (Biotronik, München, FRG) equipped with a 3.2 × 200 mm column. Amino acid analyses were used for determining the concentration of peptide solutions. They were often sufficient to assign the proper location of peptides in the known sequence of bovine β-casein (Ribadeau-Dumas et al., 1972).

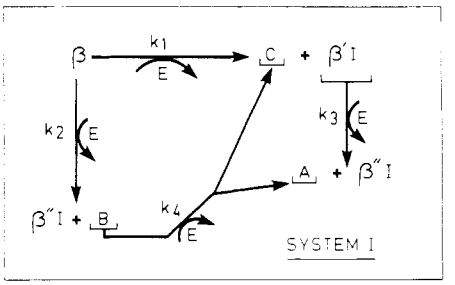

FIGURE 1: Schematic pattern of reactions studied, as related to the primary action of chymosin on β-casein: β, native β-casein; β'I, peptide 1–192 (intermediate); β''I, peptide 1–189 (end product); A, peptide 190–192 (end product); B, peptide 190–209 (intermediate); C, peptide 193–209 (end product); βI [following Creamer's nomenclature (1971)], a mixture of β'I and β''I; k_i, apparent rate constants.

Electrophoreses. They were performed on polyacrylamide–agarose (2-mm thickness), according to Uriel (1966) as modified by Gripon et al. (1975) at pH 8.6 in the presence of urea and 2-mercaptoethanol.

High-Performance Liquid Chromatography. All separations were achieved on μBondapak C_{18} columns from Waters (Milford, MA). The equipment consisted of a 720 system controller, two 6000A pumps, one U6K injector, one column (Waters), and a CE 2023 variable wavelength monitor (Cecil, Cambridge, England). The column was equilibrated in solvent A (10 mM potassium phosphate, pH 7.2), and the elution was obtained by using a biphasic linear gradient from solvent A to solvent B (60% CH_3CN and 40% A, adjusted to pH 7.2 with phosphoric acid). Both the column and solvents were kept at 40 °C in a water bath. The flow rate was 1 mL/min. The absorbance was recorded at 220 nm. The collected fractions were freeze-dried. Quantitative evaluation of peak heights was performed manually. In a preliminary trial all fractions of interest were collected. Increasing amounts of each of them were injected. In each case an excellent linearity was found when the heights were plotted against the injected amounts.

Determination of Kinetic Parameters. The different enzymic reactions involved in this study are represented in Figure 1. In the conditions where only the reaction $\beta \rightarrow C + \beta'I$ was taken into account (i.e., the other reactions were considered as negligible), the Michaelian parameters k_{cat} and K_m were determined either by using the procedure of Eisenthal & Cornish-Bowden (1974) or by integrating the Michaelis equation (see Results). A molecular weight of 35 600 was taken for chymosin (Foltmann et al., 1979).

When all the reactions of Figure 1 were taken into account (experiments at low substrate concentration), the apparent rate constants k_1–k_4 were estimated by using a computer program (available upon request from the authors). Details of the calculations are given in the supplementary material (see paragraph at end of paper regarding supplementary material).

Results

Two types of kinetic studies were performed by using RP-HPLC. In the first case, the action of chymosin was studied on micellar β-casein, i.e., at a casein concentration far above the critical micellar concentration (cmc) (Schmidt, 1982). In the second case, this concentration was below the cmc and β-casein was then monomeric.

Kinetic Study above the cmc. A casein concentration of 1% (0.34 mM) and a molar ratio E/S = 1/6000 were used. The reaction was followed by analyzing on HPLC aliquots

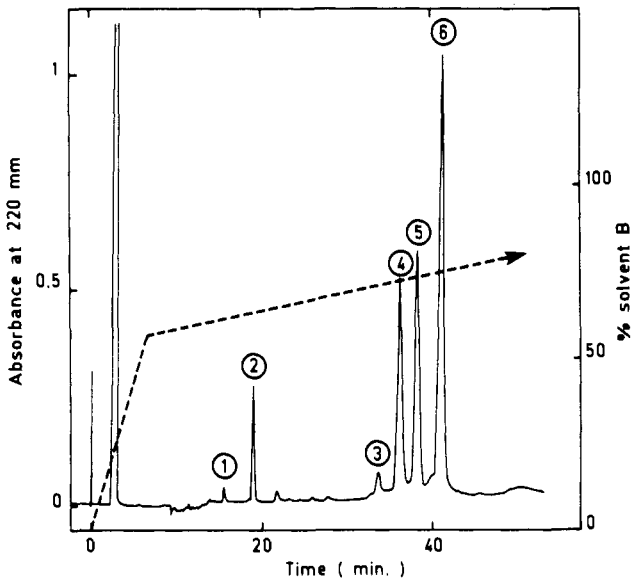

FIGURE 2: RP-HPLC elution pattern showing all molecular species that appear during chymosin action on β-casein above the cmc. There was simultaneous injection of β-casein and chymosin-treated β-casein. Conditions for the digestion and elution are described in the text. With reference to Figure 1, 1 = A, 2 = C, 3 = βII, 4 = β″I, 5 = β′I, and 6 = β. The nonretained fraction corresponds to citrate and NaN_3 present in the digestion mixture. (---) % solvent B.

taken at intervals for 3 h. Six well-resolved peaks, whose height varied with time, were observed during this reaction period. In order to identify all these components, including undegraded β-casein, 10 μL of the initial β-casein solution was injected together with 15 μL of the reaction mixture at t = 60 min. The elution profile is shown in Figure 2. Each peak was collected and analyzed. Fractions 1 and 2 were easily identified by amino acid analysis. They corresponded to peptides 190–192 and 193–209, respectively. As the other fractions contained much larger peptides, the amino acid compositions were not accurate enough to identify them unambiguously. On gel electrophoresis, fractions 3 and 6 migrated as βII and β, respectively. Fractions 4 and 5 both migrated at the level of βI, according to the nomenclature of Creamer et al. (1971).

As only βI corresponded to the peptide fraction that had to be studied, βII was no longer investigated. From amino acid analysis, it seemed to contain mainly peptide 1–166. That peak 6 was intact β-casein was verified from the retention time by chromatographing β-casein alone. Final identification of fractions 4 and 5 was obtained by analyzing the amino acids released by CPA from each of them. The results clearly showed, the amino acid compositions being known, that fractions 4 and 5 corresponded to peptides 1–189 and 1–192, respectively. As peptide 1–3, mentioned by Visser (1981), was not detected, its absence was checked as follows. The reaction mixture (E/S = 1/6000, t = 60 min) was precipitated with 12% trichloroacetic acid and centrifuged. Only fraction 1 (peptide 190–192) was detected in the supernatant by HPLC.

The quantitative evolution of the main components during a 3-h period is shown in Figure 3a. The slopes at t = 0 of the curves representing the evolutions of peptides 1–192 (β′I) and 1–189 (β″I) were proportional to their rates of appearance. From Figure 3a, a value of 9 was determined for the ratio $v_{\beta'I}/v_{\beta''I}$. It has thus been considered that, for the first 15 min, the appearance of both βII and β″I could be neglected. In these conditions the Michaelian parameters relative to the splitting of bond 192–193 could be determined. This was done by quantitative determination on HPLC of peptide 1–192 after

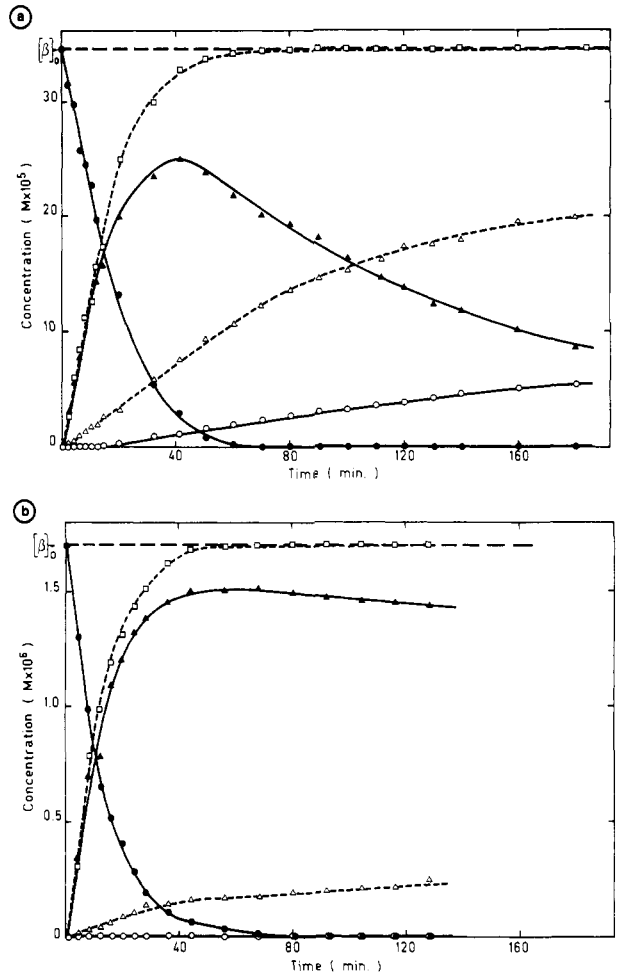

FIGURE 3: Evolution with time of the concentration of main molecular species involved in the first steps of chymosin action on β-casein: (a) above cmc; (b) below cmc. (●) β-Casein; (▲) β′I; (△) β″I; (□) C; (○) βII.

6-, 9-, 12-, and 15-min reaction times, respectively, with an enzyme concentration of 2.15×10^{-5} mM. This gave the initial rate of reaction at each substrate concentration (0.48, 0.24, 0.16, and 0.12 mM). From the results thus obtained the following values were determined by using Eisenthal–Cornish-Bowden's representation: k^1_{cat} = 1.3 s^{-1} and K^1_m = 0.075 mM.

Kinetic Study below the cmc. In this experiment, the initial substrate concentration was 0.17×10^{-2} mM. Several enzyme concentrations were tested to have a degradation of β-casein similar to that obtained above the cmc at 60 min. This was obtained with E/S = 1/60. The digest was analyzed by HPLC during a 3-h period with dansylserine as an internal standard (recorder sensitivity 10 times higher than noted previously). The evolution of the reaction products is shown in Figure 3b. Assuming that the β″I concentration was negligible over the whole reaction period, the figures obtained for the disappearance of β-casein as a function of time were used to determine the kinetic parameters relative to splitting of bond 192–193 by integrating the Michaelis equation. The following results were obtained: k^2_{cat} = 0.56 s^{-1} and K^2_m = 0.007 mM. From the data used to draw Figure 3b, the following apparent rate constants were computed: $k_1 = 1.2 \times 10^{-3}$ s^{-1}, $k_2 = 7.1 \times 10^{-5}$ s^{-1}, $k_3 = 1.5 \times 10^{-5}$ s^{-1}, and $k_4 = 4.9 \times 10^{-3}$ s^{-1}.

Discussion

From earlier publications (Pélissier et al., 1974; Creamer, 1976; Visser & Slangen, 1977; Visser, 1981) it was known that the reaction of chymosin could lead to a number of cleavages

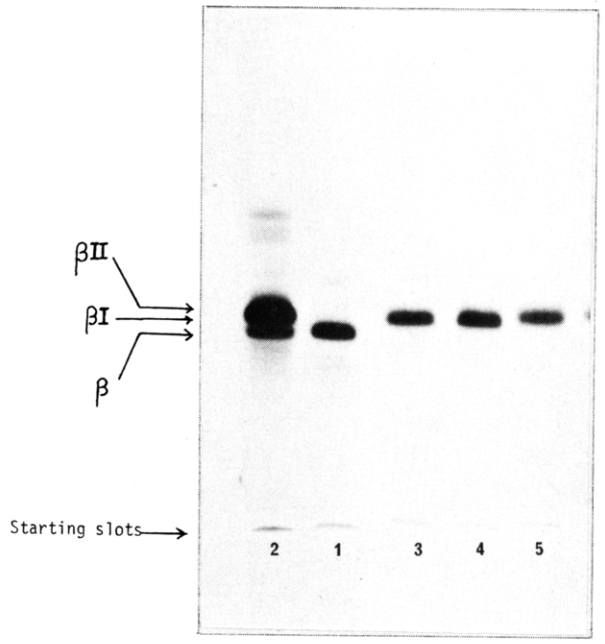

FIGURE 4: Acrylamide–agarose gel electrophoresis of a chymosin digest of β-casein above the cmc and of its peptide components separated by HPLC as in Figure 2. Conditions for the digestion are described in the text. 1, β-casein (peak 6); 2, β-casein digest; 3, peptide 1–189 (peak 4); 4, peptide 1–192 (peak 5); 5, peptide 1–166 (βII, peak 3).

in the β-casein peptide chain, even under mild reaction conditions. Cleavages at bonds 3–4, 127–128, 139–140, 163–164, 165–166, 167–168, 189–190, and 192–193 have been reported in conditions where gel electrophoresis at pH 8.6 indicated the appearance of three bands, called βI, βII, and βIII, the presence of some intact β-casein being still detected. It was shown that fraction βI corresponded to peptides 1–189 and 1–192 (it could also contain peptides 4–189 and 4–192), βII to 1–162, 1–164, 1–166, and 1–167, and βIII to 1–127 and 1–139. None of the corresponding complement peptides could be detected on the gel due to their small size. The same authors indicated that, under suitable conditions, only fraction βI could be produced. We have chosen such conditions. Indeed Figure 3b does not show the release of any βII, while this fraction is negligible for at least 20 min in the experiments carried out above the cmc (Figure 3a). This can also be observed in Figure 4.

Thus our conditions were such that only three bonds, 3–4, 189–190, and 192–193, could have been cleaved to a significant extent. We have shown that only the latter two were indeed split off. These cleavages gave three end products, peptides β″I (1–189), A (190–192), and C (193–209), that have been identified. In addition, two intermediate products could be expected: peptides β′I (1–192) and B (190–209) (Figure 1). However, peptide B never appeared under our conditions. This means that the rate of its cleavage at bond 192–193 (giving A + C) was equal to its rate of appearance. This was confirmed by the values obtained below the cmc for the two corresponding apparent rate constants $k_2 = 7.1 \times 10^{-5}$ s^{-1} (for β → β″I + B) and $k_4 = 4.9 \times 10^{-3}$ s^{-1} (for B → A + C), which differ by a factor of approximately 100.

Nothing was known about the relative sensitivities to chymosin of bonds 189–190 and 192–193 in intact β-casein. Indeed it had never been possible to separate peptides β′I and β″I, which have identical electric charges and nearly equal molecular weights. The apparent initial rate of cleavage of these bonds can be easily calculated from Figure 3 by measuring the initial rates of appearance of β″I and β′I, $v_{\beta''I}$ and $v_{\beta'I}$. Values of 9 and 20 were determined for $v_{\beta'I}/v_{\beta''I}$ above and below the cmc, respectively. This indicated that, in both cases, the cleavage of bond 192–193 is much faster than that of bond 189–190.

Table I: Comparison between Michaelian Parameters Related to Action of Chymosin on β-Casein in the Micellar or Monomeric State

	V_{max} (mM s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
micellar β-casein	2.8×10^{-5}	0.075	1.54	20.6
monomeric β-casein	0.9×10^{-5}	0.007	0.56	79.7

The kinetics of the appearance of β′I and β″I were quite different below and above the cmc (Figure 3). Below the cmc, the relative appearance of β″I was much slower and β′I, which reached a maximum at similar times in both experiments, increased to a much higher level and then decreased much more slowly with time. As the rates of disappearance of β-casein were quite similar, the probabilities for efficient collisions between enzyme and substrate were likely to be similar. The two ratios $v_{\beta'I}/v_{\beta''I}$ mentioned above differed by a factor of 2.4. They should be equal if the two reactions were independent. This was not the case. It seems therefore that splitting of bond 192–193 limits the rate of cleavage of bond 189–190, probably because of the proximity of the two bonds. The states of aggregation of β-casein are quite different on each side of the cmc. Only monomers are present below the cmc while large micellar aggregates form at the concentration used above the cmc (Schmidt, 1982). This should not affect the relative sensitivities to chymosin of the two bonds in question, as they are very close to each other and then should be equally accessible to the enzyme.

The Michaelian parameters relative to the action of chymosin on bond 192–193 in the two states of β-casein aggregation are compared in Table I. The values for k_{cat} are not significantly different whereas K_m is lower with the micellar form. This is quite logical. It is well-known that the associations of β-casein are largely due to hydrophobic interactions involving the C-terminal part of the molecule. Indeed, removing the two C-terminal residues strongly diminishes the associations (Thompson et al., 1967) while the removal of the 20 C-terminal residues abolishes these associations (Berry & Creamer, 1975). The accessibility to the enzyme of bond 192–193, which is in this area and which involves hydrophobic residues, is likely to be reduced when β-casein is in a micellar form.

In order to calculate the equations representing the evolution of the different molecular species as a function of time (Figure 1), it was necessary to have first-order reactions. This was the case with the low substrate concentration used, which was small compared to the value of K_m. Under these conditions, the reaction rates can be considered as equal to $(V_{max}/K_m)[S] = k[S]$, k being any of the four apparent rate constants (k_1–k_4) that have been calculated. Thus it is possible to check the value of k_1 by using those values determined for V_{max} and K_m corresponding to the cleavage of bond 192–193 in the monomeric form of β-casein: $V_{max} = 0.9 \times 10^{-5}$ mM s^{-1}; $K_m = 0.007$ mM; $k_1 = V_{max}/K_m = 1.3 \times 10^{-3}$ s^{-1}.

This is quite satisfactory since the value given by computation for k_1 was 1.2×10^{-3} s^{-1}. Excellent agreement was found between experimental data corresponding to Figure 3b and those calculated from the theoretical expressions of the concentrations at different reaction times obtained by resolution of the differential equation system related to system I (Figure 1) using the numeric values of k_1–k_4 determined above. Comparison of the four apparent rate constants showed that k_1 and k_4 on one hand and k_2 and k_3 on the other hand were

in the same range. This is in accordance with the fact that, in both cases, one deals with the same bond located in different substrates. If the representation of Schechter & Berger (1976) is taken for the amino acid residues surrounding the cleaved bond, that is, NH_2-...-P_4-P_3-P_2-P_1-P'_1-P'_2-P'_3-...-COOH, it is seen that only residues located between P_3 and P'_3 play a major role in the enzymic reaction. This is in accordance with the results obtained by Antonov (1977) on pepsin, an enzyme that is quite similar to chymosin. This author showed that site P_1 is the most important and that sites P_2, P_3, P'_1, and P'_2 have an appreciable influence on the catalysis. Similar conclusions were obtained by Visser (1981) for chymosin.

Acknowledgments

We thank Dr. J. C. Collin, I.N.R.A., Poligny, for the determination of chymosin activity and for checking the absence of pepsin in the chymosin preparation.

Supplementary Material Available

Additional information regarding the principle of calculation used for the determination of k_1–k_4, the apparent rate constants of the studied enzymic reactions (2 pages). Ordering information is given on any current masthead page.

Registry No. Chymosin, 9001-98-3.

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Activation of Transglutaminase during Embryonic Development†

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ABSTRACT: Incorporation of [^{3}H]putrescine into proteins was shown to increase markedly in sea urchin eggs upon fertilization. Emetine, an inhibitor of protein synthesis, had no effect on the rate of protein labeling. However, the reaction could be prevented by the addition of 2-[3-(diallylamino)-propionyl]benzothiophene, a noncompetitive inhibitor of transglutaminase, and also by dansylcadaverine, which is a substrate for transglutaminase. The inert N^{α}-dimethyl analogue of dansylcadaverine had no influence. Considering the complexity of the incorporation of the [^{3}H]putrescine tracer in this system, it was deemed essential to prove by rigorous analytical methods that the reaction was, indeed, consistent with a transglutaminase mechanism. γ-Glutamyl[^{3}H]putrescine could be recovered in 80–90% yield from the proteolytic digest of proteins from the 20-min fertilized cell. Another sign of the in vivo activity of transglutaminase was the isolation of substantial amounts of ϵ-(γ-glutamyl)lysine from proteins of sea urchin embryo, yielding a frequency value for this cross-link as high as 1 mol/400 000 g of protein in the 32-cell-stage material.

The elevation of cytoplasmic concentration of Ca^{2+} ions seems to play a central role in embryogenesis. In sea urchin, the rise in Ca^{2+} concentration occurs whithin a few seconds after fertilization. The wave of free Ca^{2+} is thought to be essential for triggering further steps in development including the cortical reaction, phosphorylation of nicotinamide adenine dinucleotide, production of hydrogen peroxide, activation of lipoxygenase, polymerization of actin, and, perhaps most significantly, promotion of a Na^+–H^+ exchange. Uptake of Na^+ and expulsion of H^+ ions, with the concomitant increase of intracellular pH, seem to be responsible for the increase in DNA and protein synthesis [for a review, see Epel (1980); also see Hamaguchi & Hiramoto (1981) and Schmidt et al. (1982)].

In the present paper, evidence is provided for the activation of transglutaminase, yet another Ca^{2+}-dependent enzyme [for

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0006-2960/84/0423-6843$01.50/0