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# α-Chymotrypsin in Plastein Synthesis

## Effect of Hydroxylated Additives on Enzyme Activity

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## ABSTRACT

The influence of different hydroxylated additives (methanol, ethyleneglycol, glycerol, erythritol, xylitol, and sorbitol) on the plastein reaction catalyzed by α-chymotrypsin has been studied at different substrate concentrations. The results obtained showed that the increase in the polyol concentration and the number of hydroxylic groups per molecule enhanced the plastein yield. However, when the substrate concentration was also increased, a decrease in this activation effect was observed. In the case of a 3*M* xylitol solution, the plastein reaction was studied by both quantification of free amino groups and gel-permeation chromatography at different substrate concentrations. The overall analysis of the results allowed us to postulate a schematic integration of all chymotryptic reactions as a general mechanism of the plastein synthesis, as well as to conclude that the presence of additives in the reaction media, as water-activity reducing agents, increase the condensation pathway of the α-chymotrypsin action on the plastein synthesis.

**Index Entries:** Plastein reaction, α-chymotrypsin; hydroxylated additives; water activity; peptide synthesis; peptide condensation; transpeptidation.

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## INTRODUCTION

Proteases are used in preparation of protein hydrolyzates for incorporation into food products. However, numerous basic studies on proteases have disclosed that these enzymes have a potential ability to catalyze several unconventional reactions (ester and amide hydrolysis, transesterification, transamidation, and condensation) in appropriate conditions. The plastein reaction, i.e., the incubation of a highly concentrated solution of protein hydrolyzate with proteases, which produces water-insoluble and gel-forming products, could be also included in this list (*1*). Synthesis of protein-like molecules by the plastein reaction is very interesting to the food industry, such as in the removal of bitterness of protein hydrolyzates (*2*), preparation of product with improved amino acids composition (*3,4*) or modification of visco-elastic properties for incorporation into different types of food (*5*).

The role of proteolytic enzymes in the plastein reaction, as well as the kinetic mechanism have been very controversial. Thus, this process usually has been considered a reversal of hydrolytic reactions carried out by a low-mol wt substrate, at an exceptionally high concentration (20–40% w/v) (*3*), where the plastein products have very high mol wt. Supporting this, Determann et al. (*6*) showed that direct condensation of some simple synthetic peptides to oligomers may occur at high substrate concentration. On the other hand, from studies of the plastein reaction catalyzed by α-chymotrypsin, Horowitz and Haurowitz (*7*) concluded that direct condensation of peptides played a minor role and that a transpeptidation mechanism was the dominating type of reaction. This hypothesis of transpeptidation mechanism, where an intermediate covalent acyl-enzyme is deacylated via aminolysis, is also supported by several authors (*8*) in the kinetically controlled peptide synthesis in organic solvent. Other authors (*9–11*) suggested that hydrophobic and ionic interactions are the more important factors in the production of the water-insoluble products called plasteins.

In the plastein reaction, the substrate concentration is the most important limiting factor in the catalytic efficiency of the protease. This parameter determines simultaneously the total free amino group concentration, as nucleophilic acyl acceptors of the enzymic complex, as well as the water activity in the reaction medium (*1,3*). In a previous paper, where the influence of the substrate concentration on the plastein reaction catalyzed by α-chymotrypsin was studied, we have observed that both hydrolytic and synthetic activities can occur simultaneously (*12*). Additionally, in the latter case, when the substrate concentration was increased, we have proved that the products obtained by the enzyme action had also changed: a decrease in the transpeptidation/condensation ratio was observed.

In order to discriminate the actual effect of the substrate concentration on the enzyme action, as nucleophilic acyl acceptors to the enzyme com-

plex and inductor of a low water content in the reaction medium, we have studied the effect of different additives in the plastein reaction catalyzed by α-chymotrypsin. Various polyhydroxylated additives (ethylene glycol, glycerol, erythritol, xylitol, and sorbitol) are known to be enzyme-stabilizing and water-activity reducing agents (*13,14*). The influence of these additives and methanol on the plastein reaction catalyzed by α-chymotrypsin has been assayed at different substrate concentrations in order to determine its influence on the enzyme action.

## MATERIALS AND METHODS

Bovine serum albumin from Sigma Chem. Co. (St. Louis, MO) was used for the preparation of substrate.

Pepsin (EC 3.4.23.1.) from porcine stomach mucosa was used as the hydrolyzing catalyst to obtain the substrate, and α-chymotrypsin (EC 3.4.21.1.) from porcine pancreas was used to catalyze the plastein reaction. Both enzymes were obtained from Sigma Chem. Co.

All remaining reagents were analytical grade.

### Substrate Preparation

A 10% (w/v) albumin solution (pH = 1.6) was treated by pepsin (1% w/w) with magnetic stirring for 48 h at 40°C. The reaction was stopped by increasing the pH to 7.0 with NaOH, and the resulting hydrolyzate solution was ultrafiltered through polysulphone membranes (10-KDa cutoff) and then concentrated in a Rotavapor to 55% (w/v). This substrate was previously characterized, showing a free amino groups concentration of 1.5 μmol $NH_3^+$/mg albumin (*12*).

### Plastein Reaction

Into an Eppendorf tube of 1 mL total volume, amounts of 100, 200, or 350 mg of the concentrated hydrolyzate of albumin were placed, and 3.5 mg of α-chymotrypsin were added. The reaction volume was adjusted to 1 mL by addition of water or aqueous polyol solution, and the reaction mixture was incubated without stirring at 40°C (*10,12,15*). Aliquots of 50 μL were extracted from the reaction mixture, previously homogenized by shaking, at different times for plastein product quantification.

### Determination of Plastein Activity

Total plastein products were determined as the fraction that precipitated in a 10% (w/v) trichloroacetic acid solution (TCA) and quantified spectrophotometrically at 280 nm by redissolving in 50% (v/v) acetic acid solution (*10,12,15*). One unit of plastein activity was defined as the amount

of enzyme that produced 1 mg of plastein per min under optimum assay conditions (*12*).

### Gel Permeation Chromatography

Gel permeation of the plastein reaction mixture was carried out in a Fast Protein Liquid Chromatography (Pharmacia Fine Chemical, Uppsala, Sweden) using a column (10×300 mm) of Superose™ 12 (Pharmacia Fine Chemical), equilibrated, and eluted with 50% (v/v) acetic acid at a flow rate of 0.1 mL/min. An aliquot of the reaction mixture (15 μL) was dissolved in 185 μL of the eluent and applied to the column. The absorbance of the column effluent was monitored at 280 nm (*12*).

### Determination of Free Amino Groups

The free amino groups of the plastein reaction mixture were determined by the formol number method (*16*), using a Metrohm (Herisau, Switzerland) pH-stat (691 pH Meter, 665 Dosimat, 614 Impulsomat) (*12*).

### Determination of Water Activity

Water activity was determined using a humidity and temperature digital indicator HUMIDAT-IC II (Novasina, Zürich, Switzerland), with a humidity sensor model BS-3(4)/PP (Novasina) at a temperature of 20°C. The humidity sensor was checked and periodically recalibrated at three points, with control saturated salt solutions (LiCl, Aw = 11.3%; $Mg(NO_3)_2$, Aw = 54.4%; and $BaCl_2$, Aw = 90.5%) for the overall measuring range.

## RESULTS AND DISCUSSION

Enzymatic hydrolytic reactions for the most part are equilibria reactions with the equilibrium shifted in the direction of the hydrolysis, which consume one or more water molecules. It is thus quite reasonable to assume that a medium with a decreased water activity, produced by high substrate concentration or water-activity reducing agents, will shift the equilibrium to the synthetic reaction (*13,17*). In this order, the effect of different hydroxylated additives containing 1-6 carbon atoms (methanol, ethylene glycol, glycerol, erythritol, xylitol, and sorbitol) has been studied in the case of the plastein reaction catalyzed by α-chymotrypsin at three different substrate concentrations (10, 20, and 35% w/v) under optimal conditions. These concentrations have been chosen because studying the plastein reaction in the absence of additives, we have proved that they are representative of three different behaviors on the biocatalytic action of the enzyme. When a 10% (w/v) substrate concentration was used, the ratio hydrolytic/synthetic reaction was shifted to hydrolysis; in the case of

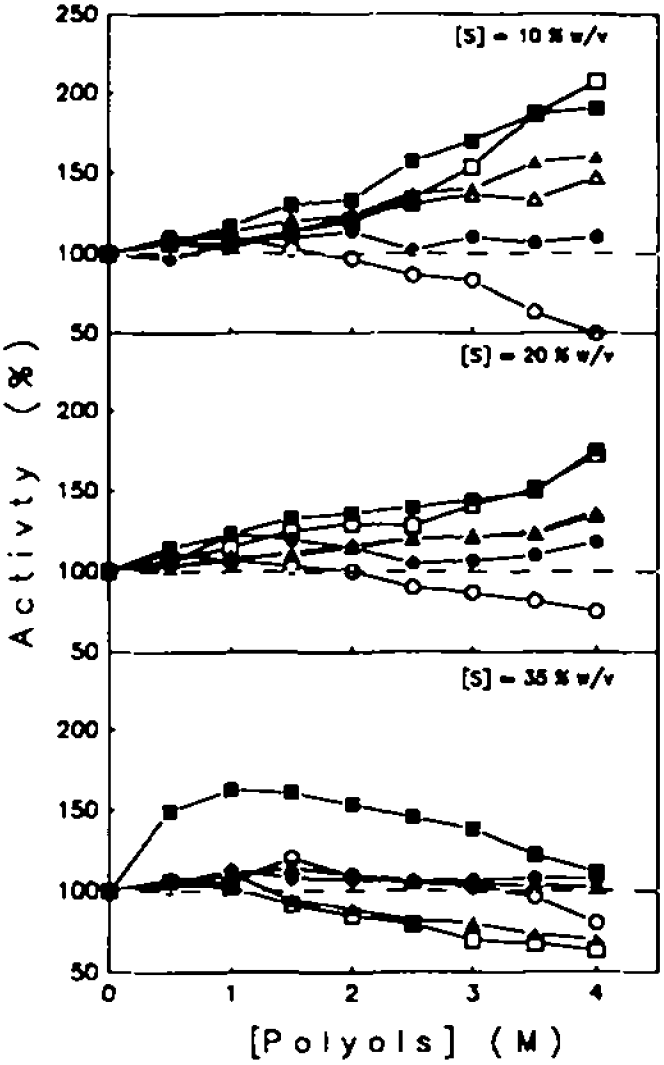



Fig. 1. Effect of hydroxylated additives concentration on plastein reaction catalyzed by α-chymotrypsin, using a peptic hydrolyzate of albumin as standard substrate, at three different concentrations (10, 20, and 35% w/v) in optimum conditions of assay (temperature, 40°C; pH, 7.0). ○ Methanol; ● Ethylene glycol; △ Glycerol; ▲ Erythritol; □ Xylitol; ■ Sorbitol.

a 20% (w/v) substrate concentration, this ratio was slightly shifted to the synthetic path: plastein products are mainly attributable to transpeptidation reaction. Finally, a 35% (w/v) substrate concentration has been chosen because the plastein production (obtained by both transpeptidation and condensation reactions) is optimum for this value (*12*).

Figure 1 shows the evolution of the plastein activity profile of the α-chymotrypsin, as a function of the additive concentration. Initially, for

the lowest substrate concentration assayed, a clear activation effect on the plastein yield was observed when the hydroxylated additive concentration was increased, except in the case of methanol. On the other hand, the increase in the substrate concentration in the reaction media involved a decrease in this activation power of the additives. When a 35% w/v substrate concentration was used, a "negative effect" was obtained in the case of methanol, erythritol, and xylitol. Ethylene glycol and glycerol had no effect, whereas sorbitol exhibited a positive effect. However, these results must be analyzed separately, as a function of the substrate concentration, for each polyhydroxylic additive.

In Fig. 2, the effect of the different polyols is analyzed as a function of the number of hydroxylic groups per molecule of additive. At low substrate concentration, this figure shows that the increase in the mol wt of the additive involved an increase in the plastein activity. This activation effect was reversed at the highest substrate concentration. However, in this figure, two exceptions can be observed: methanol and sorbitol. In the case of methanol, the increase in the substrate concentration involved a decrease in the deactivation effect observed in all cases. This fact could be a consequence of a particular deactivation capability of this organic solvent for α-chymotrypsin, also reported by Kise et al. (*18*), when transesterification and peptide synthesis reactions were carried out with methanol in the reaction media. On the other hand, an unexpected activation effect was observed for the sorbitol when the highest substrate concentration was assayed. This particular behavior should be explained as a consequence of the specific interactions between sorbitol and substrate, inducing conformational changes, or between sorbitol and the enzyme modifying its catalytic activity (*19*).

An overall analysis of the effect of polyols on the plastein reaction catalyzed by α-chymotrypsin takes into account both overall and particular effects of each additive in the catalytic capability of the enzyme, as well as the microenvironmental conditions of reaction. This overall effect is depicted in Fig. 3 as a function of the overall concentration of hydroxylic groups in the reaction media, and it is independent of the nature of the additive. The more the substrate is concentrated, the more the relative activation effect is reduced. Moreover, in the case of the most concentrated substrate solution, this effect is finally inversed. This unexpected change in the behavior of the reaction could be attributed at the same time to the observed rise of the viscosity in the reaction media, producing a limitation in the microenvironmental conditions for the diffusion of the substrate and products (*12*), and also to a change of the tridimensional structure of the enzyme owing to the very low water activity (*19*). These facts, in addition to gelation and formation of the insoluble material in the reaction mixture, should logically be related to the observed reduction of the activation power of the hydroxylated additives in the plastein yield of reaction, when the substrate concentration was increased.

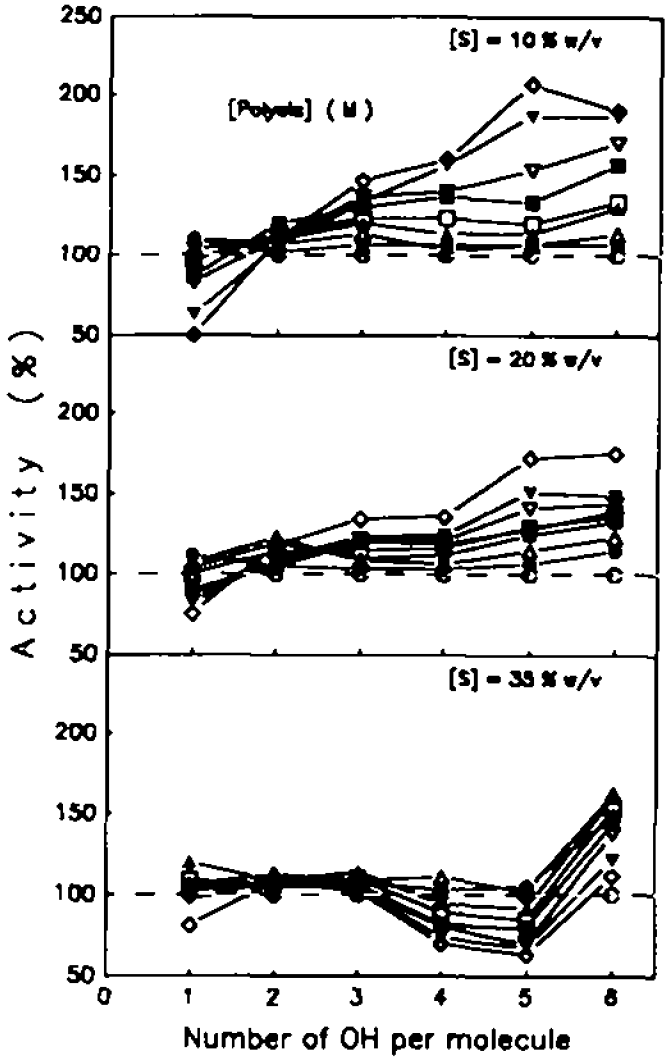



Fig. 2. Effect of length of hydroxylated additive molecule on plastein reaction catalyzed by α-chymotrypsin using a peptic hydrolyzate of albumin as standard substrate, at three different concentrations (10, 20, and 35% w/v) in optimum conditions of assay (temperature, 40°C, pH, 7.0). ○ 0.0; ● 0.5; △ 1.0; ▲ 1.5; □ 2.0; ■ 2.5; ▽ 3.0; ▼ 3.5; ◇ 4.0.

It has been reported that a substantial reduction of the water activity of the medium is needed before the equilibrium of a hydrolytic reaction will be reversed. The water activity ($A_w$) is defined as the ratio of the water vapor pressure over reaction medium to that over pure water (*17*). Then, we have measured the water activity of our reaction medium with or without additives at various substrate concentrations. Figure 4 represents the evolution of the specific activity of α-chymotrypsin in plastein synthesis vs water activity. As it can be seen, the effect of the different hydroxylated

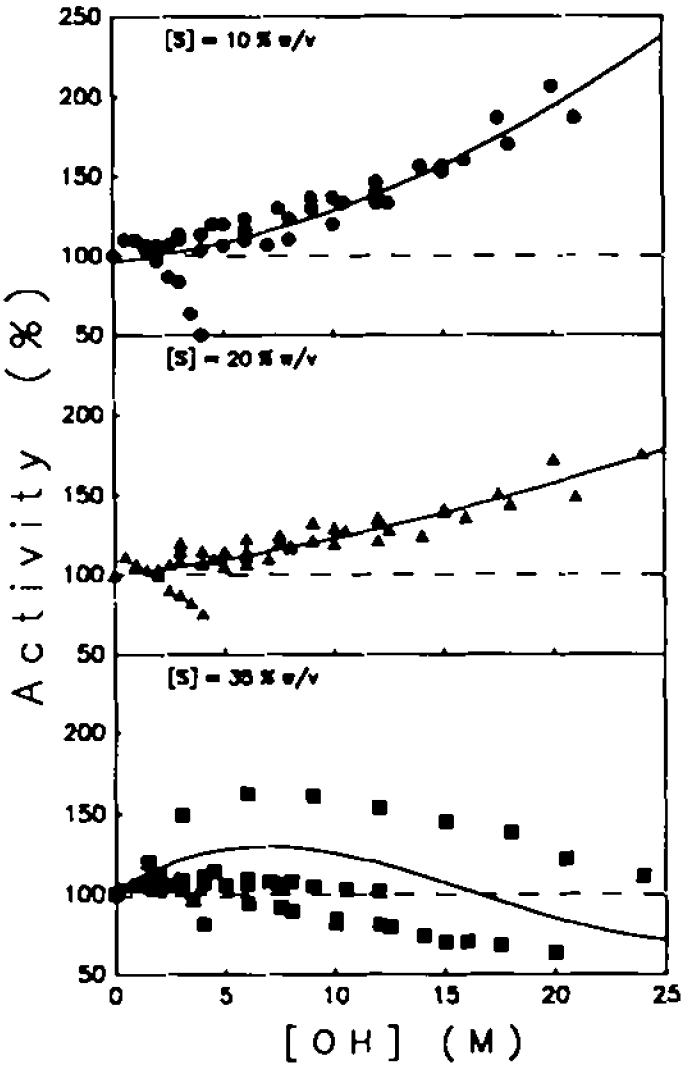



Fig. 3. Effect of total hydroxylic groups concentration of additives on plastein reaction catalyzed by α-chymotrypsin, using a peptic hydrolyzate of albumin as standard substrate, at three different concentrations (10, 20, and 35% w/v) in optimum conditions of assay (temperature, 40°C; pH, 7.0)

additives used [methanol (C1), ethylene glycol (C2), glycerol (C3), erythritol (C4), xylitol (C5), and sorbitol (C6)] cannot be clearly differentiated for each substrate concentration. On the other hand, an increase in the substrate concentration implied an increase in the initial specific activity without additive (*12*). Furthermore, the decrease in $A_w$ is followed by a slight increase in this specific activity. It is evident from this figure that $A_w$ is not the "motor" of the reaction and that substrate concentration is the determinant factor. In our case, plastein synthesis should be regarded as

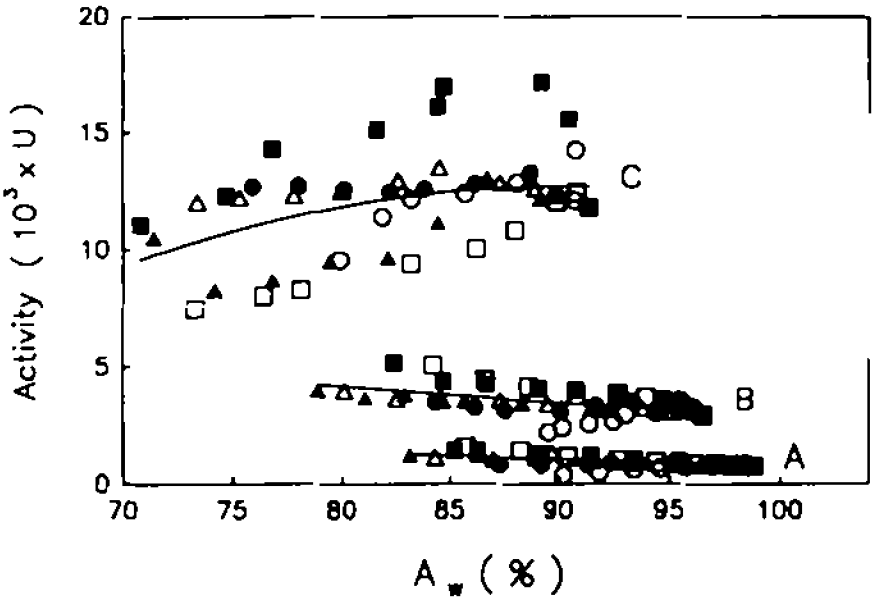



Fig. 4. Effect of the water activity (Aw) into the reaction media on the activity of α-chymotrypsin in plastein synthesis, using a peptic hydrolyzate of albumin as standard substrate, at three different concentrations (A = 10, B - 20, and C - 35% w/v, respectively). Different hydroxylated additives (C1: methanol; C2: ethylene glycol, C3: glycerol; C4: erythritol; C5: xylitol; and C6: sorbitol) at several concentration (0-4*M*) were used as water activity depressors into the reaction medium. ○ C1; ● C2; △ C3; ▲ C4, □ C5; ■ C6.

being "pushed" by a high concentration of reactants, rather than being "pulled" by low water activity (*17*). Moreover, this low water content determines an increase in the partial concentration of free amino groups with respect to water, favoring the synthetic pathway of the equilibrium-controlled reaction of peptide synthesis that can occur. However, the decrease in $A_w$ is not sufficient condition for water mass action to give an increase in the yield of synthetic product (*20*).

On the other hand, another way to study the effect of these additives on the plastein synthesis catalysed by α-chymotrypsin should be carried out by the analysis of the plastein products. In our case, a complex mixture of oligopeptides was used as substrate. Thus, this study can be made by both gel permeation chromatography and quantification of free amino groups in the reaction medium with time. In this order, the effect of a 3*M* xylitol solution was chosen as representative example to study the effect of additives by both methods and to compare with a reaction medium without any additive. Figure 5 shows the different gel permeation chromatography profiles at several reaction times, with and without xylitol (3*M*) in the reaction medium, at three different substrate concentrations, using

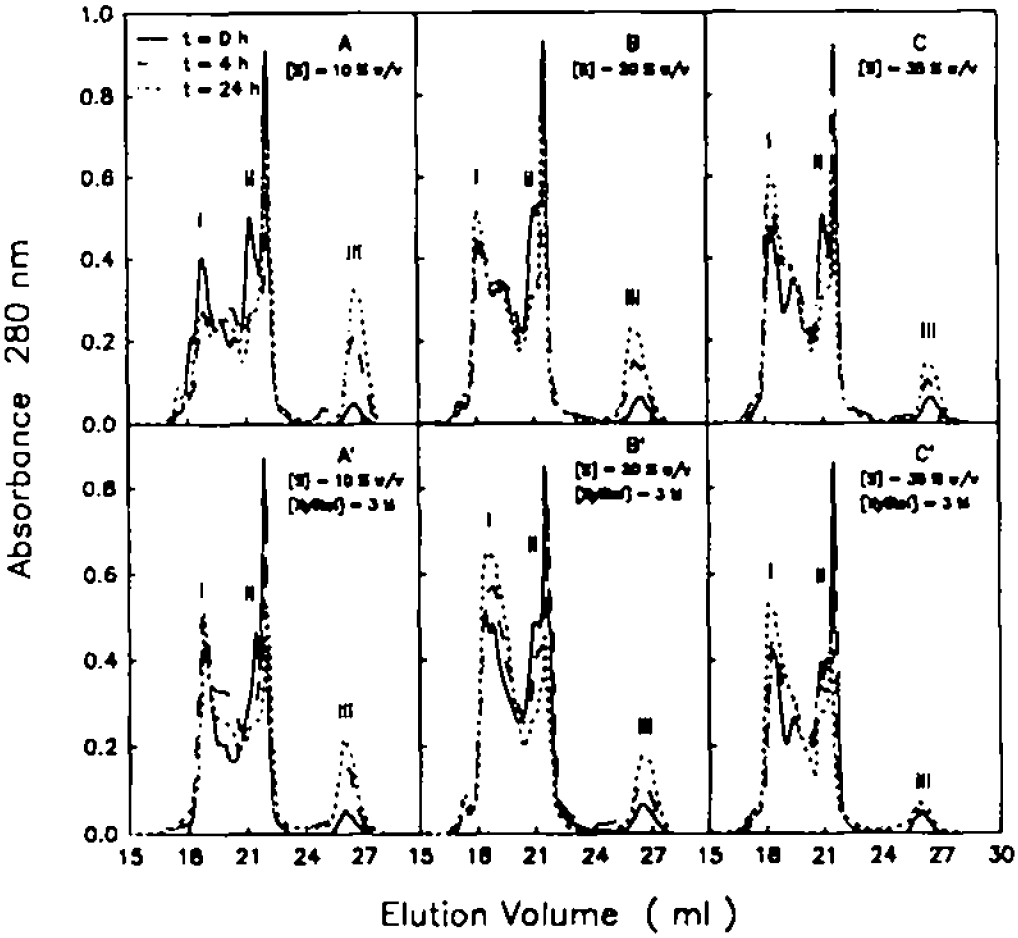



Fig. 5. Gel permeation chromatograms of the plastein reaction mixture with time catalyzed by α-chymotrypsin, using a peptic hydrolyzate of albumin as standard substrate, at three different concentrations (10, 20, and 35 w/v), with or without the presence of a 3*M* xylitol concentration in reaction media, in optimum conditions of assay (temperature, 40°C; pH, 7.0). A FPLC system with a Superose 12™ as column and a 50% (v/v) acetic acid solution as mobile phase at 0.1 mL/min were used as chromatographic conditions.

a Superose 12™ (Pharmacia) as column. In all cases, no thermal deactivation process on the enzyme action was observed during the period of reaction studied (*12*). The distribution of the material in each reaction mixture was eluted in several fractions clearly differentiated. We have chosen to discuss the three main fractions: fraction I: eluted between 18–20 mL elution volume; fraction II (21–23 mL elution volume), and fraction III (25–27 mL elution volume). As a function of time, the profiles of the fraction I and II were shown to change, with an increase in fraction I and a decrease in fraction II, as a consequence of the synthetic activity of

the α-chymotrypsin. However, fraction III, corresponding to the low-mol size peptides, was also increased with reaction time. This simultaneous increase in the high (I) and low (III) peptide fraction was explained by a transpeptidation mechanism (*9,12*). Moreover, the increase in the substrate concentration involved a concomitant increase in the high peptide fraction (I) and a decrease in the production of the low peptide fraction (III). In the catalytic action of the α-chymotrypsin, this fact implied clearly that a decrease in the hydrolysis/synthesis product ratio occurred, when the substrate concentration increased. Furthermore, the presence of xylitol in the reaction media produced a similar effect to the increase in substrate concentration: the production of the low oligopeptide fraction (III) is reduced (Fig. 5). This fact could also be ascribed to an increase in this condensation/transpeptidation product ratio of the enzyme action. The elution profiles of the fraction III, in the case of Fig. 5B (substrate concentration = 20% w/v) correspond to the elution profiles of fraction III in the case of Fig. 5A' (substrate concentration = 10% w/v) The same result is observed when comparing Fig. 5C and Fig. 5B' and it was also obtained by comparison between Fig. 5C' and a plastein reaction assayed at 42.5% w/v of substrate concentration. Thus, the addition of these additives corresponds to an increase in the substrate concentration, but their role is only as a water-activity reducing agent. Then, the reaction seems to be "pulled" by the low water content.

On the other hand, the concentration profile of total free amino groups with reaction time was also studied for the abovementioned conditions, in order to confirm this conclusion (Fig. 6). An increase in this parameter indicates that hydrolytic and synthetic transpeptidation pathways occurred at the same time, whereas a decrease in it implies clearly that condensation was favorably present. As can be seen, Fig. 6 shows how the increase in substrate concentration implied a reduction in the level of free amino groups concentration with reaction time. Moreover, in all cases, the presence of xylitol produced an additional decrease in the final level of free amino groups concentration of the reaction media In the case of the highest substrate concentration (35% w/v), the addition of 3*M* xylitol induced an important decrease in the free amino groups concentration, related to the enhanced condensation pathway and similar to the effect of a 42.5% w/v initial substrate concentration without additive (*12*).

These results allowed us to postulate an integration of all chymotryptic reactions as a general mechanism responsible for the plastein synthesis, as a kinetically and equilibrium controlled process of peptide synthesis, summarized in the Scheme I. These catalytic pathways of α-chymotrypsin in plastein synthesis take into account the different types of reactions: hydrolytic, transpeptidation, and condensation reactions. Moreover, it permits us to discriminate the role of the concentration of the two reactants: substrate and water. For instance, in α-chymotrypsin-catalyzed peptide hydrolysis, two covalently linked enzyme-peptide intermediate,

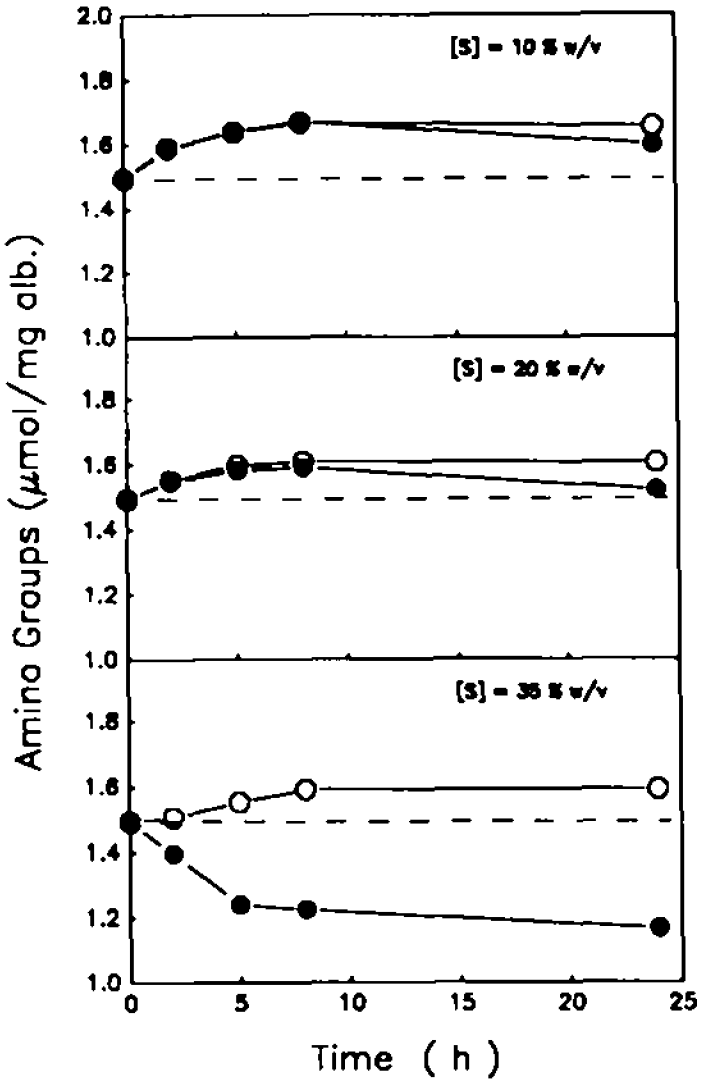
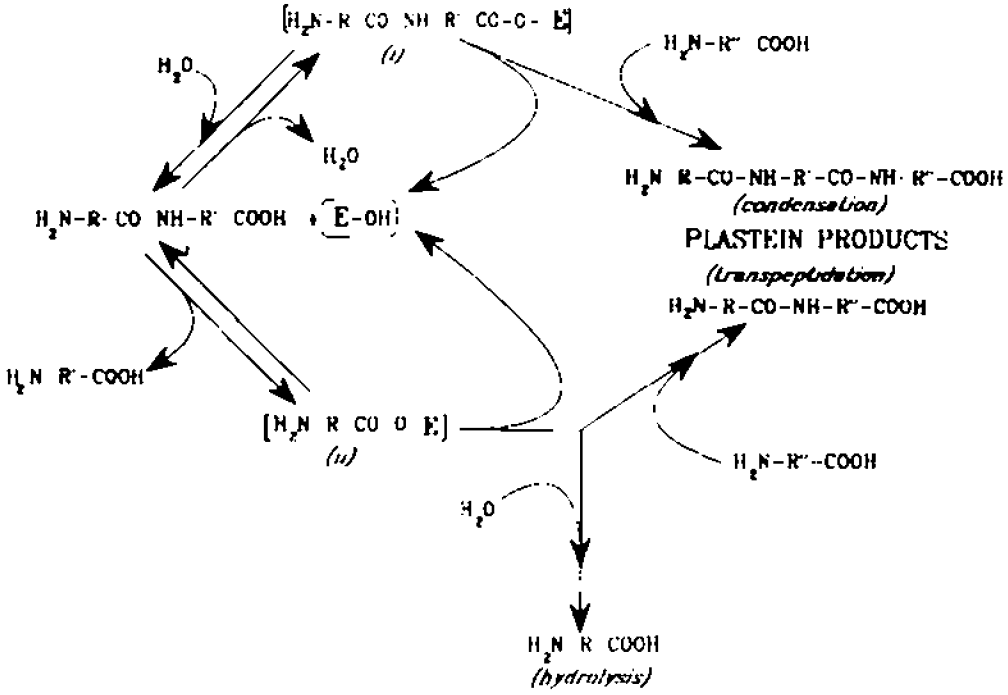



Fig. 6. Reaction profiles of free amino groups concentration in the plastein reaction catalised by α-chymotrypsin, at three different substrate concentrations (10, 20, and 35% w/v), with or without the presence of a 3*M* xylitol concentration in the reaction media, in optimum conditions of assay (temperature, 40°C; pH, 7.0). (○) Without Xylitol; (●) [Xylitol] = 3*M*.

(i) and (ii), can be formed. In a highly hydrated reaction media, the two acyl-enzyme complexes are susceptible to a nucleophilic attack by water, resulting in hydrolysis of both intermediates, and allowing the peptide hydrolysis products. Nevertheless, the increase in the substrate concentration, which directly implied an increase in the free amino groups/water relationship concentration (by reducing the water content), involves a




Scheme 1. Hypothesis of the general mechanism of plastein synthesis.

competitive nucleophilic power of this amino group to break out the acyl-enzyme complex to a synthetic pathway. Thus, the essentially irreversible peptide bond formation can be regarded as kinetically controlled synthesis, where rapid accumulation of the covalently linked intermediate (ii) and preferential nucleophilic attack by the peptide rather than water are essential, resulting in the transpeptidation products. The acyl-enzyme complex (i) is very susceptible to water attack, and the peptide synthesis reaction via condensation pathway, a water-producing reaction pathway, could only occur at a very high peptide-substrate concentration or low water-activity in the reaction media. Additionally to the biocatalytic action of the enzyme in the plastein reaction, the apparent equilibrium is shifted toward synthesis because the soluble product concentration in the reaction mixture is reduced by precipitation (*8,17*).

As a function of this postulated mechanism of the plastein reaction, the quantity of products of the α-chymotrypsin action are obtained in the order: hydrolysis < transpeptidation < condensation, when the water-activity was decreased and/or the water-competitive nucleophile (free amino groups of low-size peptides) concentration was increased. Thus, the presence of the polyols in the reaction media, which involves a reduction on water activity, favors the shift of the reaction products to the condensation pathway (*20*).

## CONCLUSIONS

The effect of 1-6 polyhydroxylic additives on the plastein reaction catalyzed by α-chymotrypsin was studied at different substrate concentrations. For the lowest substrate concentration, the increase in the mol wt or polyol concentration clearly increase the plastein activity of the enzyme. In all cases, the increase in the substrate concentration involved a decrease in this activation effect.

The results obtained from this work allow us to conclude that in the plastein reaction catalyzed by α-chymotrypsin, all possible biocatalytic pathways: hydrolysis, synthetic-transpeptidation, and synthetic-condensation can occur simultaneously. The increase in the acyl-nucleophilic acceptors concentration, as free amino groups, and the reduction of the water-activity, by the presence of polyols in the reaction media, allow us to enhance the condensation/transpeptidation plastein product ratio.

This study shows the possibility to make up synthetic reactions by the condensation of specific peptides catalyzed by proteolytic enzymes in aqueous media at relatively low substrate concentration. The reaction is shifted to this pathway by the addition of water-activity reducing agents (polyhydric alcohols), showing clearly how a change in the microenvironment of the enzyme can modify its catalytic behavior.

## ACKNOWLEDGMENTS

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