It was reported (15) that for ferricyanide ions diffusing in solutions of xanthan gum, the diffusion coefficient was decreased as much as 20-fold and was decreased as the shear rate was increased. It is thought that the shear causes the xanthan gum molecule to extend, exposing charge sites, and then the interaction between the solute and the charge sites changes diffusivity.

In addition, the viscosity measured by the viscometer may not represent the actual resistance to solute molecule transport. The values of the η_D of solutions of xanthan gum were calculated by Eq. 1. The relation of dissolution rate to viscosity of solutions of xanthan gum shown in Fig. 7 is not the same as the relation of dissolution rate to viscosity of solutions of the nonionic polymers. The difference may be caused by the interaction between the benzoic acid molecule and the anionic segments of the xanthan gum.

It appears that for viscosity-enhancing polymers of similar chemical and nonionic nature, a relationship may be expressed between the viscosity of the dissolution medium and the dissolution rate of a solid. However, if the chemical or ionic nature of the polymers is different, additional factors that influence dissolution rate are introduced and must be considered.

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Synthesis and Catalytic Activity of Poly-L-Histidyl-L-Aspartyl-L-Seryl-Glycine

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Abstract □ Poly(His-Asp-Ser-Gly) was synthesized from the fully protected tetrapeptide active ester hydrochloride, which was prepared by stepwise coupling, using pentachlorophenyl ester and mixed anhydride methods. Complete deprotection of the protected tetrapeptide polymer was achieved by using 90% trifluoroacetic acid. The free polymer was dialyzed for 24 hr using a membrane (which retains molecules with molecular weights >5000). The catalytic activity was determined by studying the hydrolysis of *p*-nitrophenyl acetate in 0.2 *M* phosphate buffer (pH 7.4) at 37°. The catalytic coefficient of the dialyzed polymer was found to be 138 liters/mole/min.

Keyphrases □ Poly-L-His-L-Asp-L-Ser-Gly—synthesis and catalytic activity, polymer, pentachlorophenyl ester, hydrolysis □ Polymer—synthesis and catalytic activity of poly-L-His-L-Asp-L-Ser-Gly, pentachlorophenyl ester, hydrolysis □ Hydrolysis—synthesis and catalytic activity of poly-L-His-L-Asp-L-Ser-Gly, pentachlorophenyl ester, polymer

The use of poly(amino acids) as esterase models for structural and mechanistic problems of proteolytic enzymes has been very useful (1, 2). The poly(amino acids) that have been used for this purpose include homopolymers, copolymers, and sequential polypeptides. Sequential polypeptides provide one of the best means to study the factors affecting the folding of various enzymes, since they permit the placement of specific side chains at specific locations on a polypeptide backbone. Thus, suitable folding brings important side chain functionalities required for the activity into closer proximity, even though these amino acids lie at different distances in the enzyme sequences.

Considerable attention has been focused recently on the role played by L-histidine and L-serine in the active site of chymotrypsin and other proteolytic enzymes toward the hydrolysis of various esters such as *p*-nitrophenyl acetate (3, 4). Synthesis and catalytic activity of a number of peptides incorporating histidine and serine have been recently reported (5–7) as esterase models. The synthesis of the pentapeptide; L-Ser-γ-aminobutyryl-L-His-γ-aminobutyryl-L-Asp which had a catalytic coefficient of 147 liters/mole/min has been reported (5). The synthesis of a relatively more potent esterase model, L-His-Gly-L-Asp-L-Ser-L-Phe which had a catalytic coefficient of 179 liters/mole/min has also been reported (6). The comparison of the catalytic activity of various peptide esterase models led to the conclusion that peptides that incorporated L-aspartic acid in addition to L-histidine and L-serine showed considerable increase in catalytic activity (7). For example, L-His-L-Ala-L-Asp-Gly-L-Ser showed a catalytic activity of 210 liters/mole/min. The corresponding pentapeptide, L-His-L-Ala-L-Glu-Gly-L-Ser, where aspartic acid was replaced by glutamic acid, had a catalytic activity of 87

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Table I—Analytical Data of Peptide Intermediates

Compound	mp	$[\alpha]_D^{20}$	IR Peaks	Microanalysis (calc) act / (calc) C	H	N	Formula
I	161–163°	+7.9 (1.5, dioxane)	5.6μm C(=O)—O—C_6Cl_5 6.05μm Amide I 6.55μm Amide II	45.88 / (45.96)	3.81 / (3.83)	4.68 / (4.66)	$C_{23}H_{23}N_2O_6Cl_5$
II	159–160°	+12.50 (1.5 dioxane)	5.6μm C(=O)—O—C_6Cl_5 6.05μm Amide I 6.55μm Amide II	35.70 / (35.79)	3.60 / (3.57)	5.59 / (5.57)	$C_{15}H_{18}N_2O_4Cl_6$
III	143–144°	+8.4 (1.0 dioxane)	5.6μm C(=O)—O—C_6Cl_5 6.05μm Amide I 6.55μm Amide II	48.28 / (48.22)	4.62 / (4.67)	5.50 / (5.45)	$C_{31}H_{36}N_3O_9Cl_5$
IV	189–190°	−6.7 (1.0, dimethyl-formamide)	5.6μm C(=O)—O—C_6Cl_5 6.05μm Amide I 6.55μm Amide II	41.04 / (40.95)	4.56 / (4.60)	6.20 / (6.23)	$C_{23}H_{31}N_3O_7Cl_6$
V	138–139°	−14.50 (1.0 dimethyl-formamide)	5.6μm C(=O)—O—C_6Cl_5 6.05μm Amide I 6.55μm Amide II	49.89 / (49.98)	4.99 / (5.06)	8.39 / (8.33)	$C_{42}H_{51}N_6O_{12}Cl_5$
VI	179–180°	−9.80 (1.0, dimethyl-formamide)	5.6μm C(=O)—O—C_6Cl_5 6.05μm Amide I 6.55μm Amide II	44.82 / (44.79)	5.09 / (5.05)	9.14 / (9.22)	$C_{34}H_{46}N_6O_{10}Cl_6$
VIII Dialysed	229–230° decomp.	−17.05 (0.05, water)		45.29 / (45.46)	4.97 / (5.05)	21.07 / (21.21)	H-$C_{15}H_{20}N_6O_7$-OH

liters/mole/min. Generally, the incorporation of aspartic acid instead of glutamic acid in the peptide sequence led to a threefold increase in catalytic activity. The increased catalytic activity of peptide esterase models containing aspartic acid may be justified on the basis that in the case of chymotrypsin, aspartic acid occupies position 194, which is adjacent to postulated active serine at position 195. The same is true of trypsin, in which case aspartic acid is at position 184, adjacent to active serine at position 185.

While the catalytic activity of a number of peptide esterase models incorporating L-histidyl, L-serine, and L-aspartyl showed an appreciable increase in activity compared with previously reported peptide esterase models, the catalytic activity of these esterase models was considerably lower than that of chymotrypsin. It was, therefore, considered worthwhile to carry out the synthesis of a sequential polypeptide incorporating L-histidyl, L-aspartyl, L-serine, and glycine.

EXPERIMENTAL

All melting points are uncorrected and were taken on a capillary melting point apparatus[1]. Results of microanalyses[2] were within ±0.4% of theoretical and are shown in Table I. Unless otherwise stated, IR spectra were determined in potassium bromide pellets on an IR spectrophotometer[3]. UV spectra were determined on UV spectrophotometer[4]. Optical rotations were measured with a precision polarimeter[5]. Before incorporation all intermediates were purified to homogeneity on TLC in the following solvent systems: (a) butanol–glacial acetic acid–water (4:1:1); (b) butanol–glacial acetic acid–water–pyridine (15:3:12:10); (c) hexane–ethyl acetate–glacial acetic acid (20:10:1; and (d) heptane–*tert*-butyl alcohol–glacial acetic acid (3:2:1). Ninhydrin was used to detect free amino group-containing peptide intermediates. Fluorescent plates were used so that ninhydrin-negative peptides could be visualized. Dialyses were carried out in an ultrafiltration apparatus[6].

All solvents were analytical grade and were distilled before use. Tetrahydrofuran was refluxed with sodium hydroxide and distilled over sodium strips. All intermediates were dried over sodium hydroxide and phosphorus pentoxide in a vacuum dessicator. Triethylamine was purified by storing over *N*-carbobenzoxyglycine pentachlorophenyl ester to remove primary and secondary amines to avoid termination of polymerization reactions (8).

N-Carbobenzoxy-(*O* - *tert* - butyl)-Ser-Gly-pentachlorophenyl Ester (I)—*N*-Carbobenzoxy-*O*-*tert*-butyl-Ser (9) (10 g, 34 mmoles) was dissolved in tetrahydrofuran (50 ml). *N*-Methylmorpholine (3.4 ml, 34 mmoles) was added and the solution was cooled to 20°. Isobutyl chlorocarbonate (4.7 ml, 37 mmoles) was added to this solution in two portions with constant stirring. After stirring for 30 min, glycine pentachlorophenyl ester hydrochloride (8) (11.03 g, 34 mmoles) was added in small portions as a suspension in tetrahydrofuran. *N*-Methylmorpholine (3.4 ml, 34 mmoles) diluted with tetrahydrofuran (20 ml) was added dropwise over a period of 1 hr. The reaction mixture was stirred in an icebath and gradually allowed to reach room temperature overnight. The precipitated material was removed by filtration and the filtrate was evaporated under vacuum.

The residual oil was dissolved in ethyl acetate (50 ml) and washed twice with 5% citric acid solution (50 ml), once with water (50 ml), twice with 2 *N* $KHCO_3$ solution (50 ml), and twice with water (50 ml). The combined aqueous washes were washed with a portion of fresh ethyl acetate. The ethyl acetate solutions were combined, dried with anhydrous magnesium sulfate, filtered, and the solvent was evaporated under vacuum. The residual oil was crystalized from hexane by cooling at dry ice temperature. The resulting solid was filtered, air dried, and recrystallized from aqueous ethanol by seeding with solid from the hexane product. The product was dried over phosphorus pentoxide and sodium hydroxide pellets (13.1 g), 64% yield.

[1] Thomas-Hoover Uni-melt.
[2] Schwarzkopf Microanalytical Lab., Woodside, N.Y.
[3] Perkin-Elmer model 257.
[4] Perkin-Elmer Hitachi 200.
[5] Rudolph model 50.
[6] DM5 membrane, Amicon Corp.

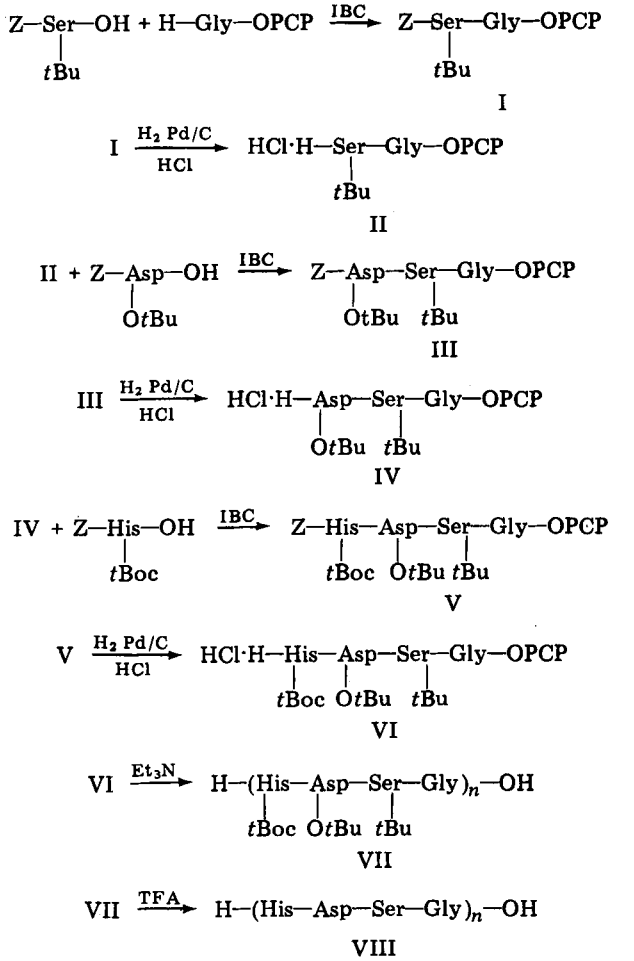
(*O-tert*-Butyl)-Ser-Gly-pentachlorophenyl Ester Hydrochloride (II)—Palladium–charcoal catalyst (10%, 0.5 g) suspended in glacial acetic acid (1 ml) and anhydrous methanol (20 ml) was hydrogenated at atmospheric pressure. Dry methanol containing 0.140 g of hydrogen chloride gas per milliliter (0.45 ml, 1.7 mmoles) was added to the hydrogenated catalyst, and the hydrogenation was continued until no further uptake of hydrogen occurred. A suspension of active ester (I, 1.0 g, 1.67 mmoles) in methanol was added to the reaction mixture and the hydrogenation was carried out until no further uptake of hydrogen occurred (10 min). The reaction mixture was filtered to remove the catalyst and the filtrate was concentrated under reduced pressure to a volume of 2 ml. Anhydrous ether was added and the hydrochloride was crystallized, (0.78 g), 93% yield.

***N*-Carbobenzoxy-(*O*-*tert*-butyl)-Asp-(*O*-*tert*-butyl)-Ser-Gly-pentachlorophenyl Ester (III)**—*N*-Carbobenzoxy(*O*-*tert*-butyl) aspartic acid (10) (3.23 g, 0.01 mole) and *N*-methylmorpholine (1.0 ml, 0.01 mole) in tetrahydrofuran (50 ml) was coupled to pentachlorophenyl ester hydrochloride (II, 5.03 g, 0.01 mole). The residue obtained after evaporation of the ethyl acetate was crystallized from ethyl acetate–ether–petroleum ether to give *N*-protected tripeptide active ester (III) (2.14 g), yield 67%.

(*O*-*tert*-Butyl)-Asp-(*O*-*tert*-butyl)-Ser-Gly-pentachlorophenyl Ester Hydrochloride (IV)—Active ester (II, 1.0 g, 1.3 mmoles) was hydrogenated to give hydrochloride (IV, 0.69 g), yield 78%.

***N*-Carbobenzoxy(imbutyloxycarbonyl)-His-(*O*-*tert*-butyl)-Asp-(*O*-*tert*-butyl)-Ser-Gly-pentachlorophenyl Ester (V)**—*N*-carbobenzoxy(imbutyloxycarbonyl)-histidine (11) (2.6 g, 6.7 mmoles) in tetrahydrofuran (50 ml) was coupled to tripeptide ester hydrochloride (IV, 4.5 g, 6.8 mmoles). The product obtained after evaporation of the ethyl acetate, was crystallized from ethyl acetate–ether–petroleum ether to give compound V (4.35 g), yield 71%.

(Imbutyloxycarbonyl) -His- (*O*-*tert*-butyl) -Asp- (*O*-*tert*-butyl)-Ser-Gly-pentachlorophenyl Ester Hydrochloride (VI)—A suspension of active ester (V, 1.0 g, 0.99 mmole) was hydrogenated to produce the tetrapeptide hydrochloride (VI, 0.53 g), yield 64%.



Scheme I—tBu, tert-butyloxy; OPCP, Pentachlorophenoxy; tBoc, tert-butyloxycarbonyl; IBC, Isobutylchloroformate; TFA, Trifluoroacetic Acid.

Poly(imbutyloxycarbonyl) -His- (*O* -*tert* -butyl)-Asp-(*O* -*tert*-butyl)-Ser-Gly (VII)—Fine powder of tetrapeptide active ester hydrochloride (VI, 2.5 g, 2.8 mmoles) was slowly added to dimethylformamide at room temperature with continuous shaking. To this suspension was added purified triethylamine (0.78 ml, 5.6 mmoles) and shaking was continued at room temperature for 48 hr. After trituration with anhydrous ether (20 ml), the reaction mixture was centrifuged, and the ether layer was decanted. This step was repeated three more times, and the residue was dried under a stream of nitrogen. Further drying of the residue was carried out in a vacuum desiccator over phosphorus pentoxide to yield 1.1 g, the protected polymer (VII, 1.1 g).

Poly(His-Asp-Ser-Gly) (VIII)—A solution of protected polymer (VII, 0.9 g) in 90% trifluoroacetic acid (4 ml) was kept at room temperature for 1 hr and the product was precipitated with ether to yield 0.5 g of unprotected polymer (VIII).

Dialysis of Unprotected Sequential Polymer—Crude unprotected polymer (VIII, 450 mg) was dissolved in water (10 ml) and dialyzed for 14 hr in a continuous filtration apparatus[6]. The concentrates were lyophilized over a period of 24 hr to give 180 mg of a white fluffy solid.

Optical Purity of Poly(His-Asp-Ser-Gly)—A solution of the free dialyzed polymer (VIII, 39.4 mg) in 6 *N* HCl (0.5 ml) and glacial acetic acid (0.5 ml) was refluxed for 24 hr in an atmosphere of nitrogen. The clear hydrolysate was evaporated to dryness under reduced pressure. The residue was dissolved in 5 *N* HCl so that the final volume was 2 ml (50 m*M*). A control sample using histidine (15.5 mg), aspartic acid (13.3 mg), serine (10.5 mg), and glycine (7.5 mg) was treated similarly with 6 *N* HCl and glacial acetic acid, then evaporated and dissolved in 5 *N* HCl as above (50 m*M*). Observed rotations of both were measured at 25°; control was +0.830° and polymer-derived material was +0.831°.

Determination of Catalytic Activity—For the measurement of catalytic activity of a sequential polypeptide (VIII) a modified method (3) was used. The rate of hydrolysis of *p*-nitrophenylacetate was calculated from the rate of appearance of the *p*-nitrophenoxide ion as measured by the increase in optical density at 400 μm. The catalytic coefficient k_2 is defined as $k_1 - k_w/C$, where k_1 is the observed first-order rate constant of hydrolysis measured in the presence of catalyst, k_w is the constant in absence of catalyst (both expressed in min^{-1}), and C is the molar concentration of the catalyst.

RESULTS AND DISCUSSION

While a number of approaches have been utilized for the synthesis of sequential polypeptides, there have been problems encountered with racemization, intramolecular cyclization, and the low degree of polymerization.

In order to limit some of these problems a suitable synthetic method for simple sequential polypeptides was reported (12). In this approach, *C*-terminal activation was provided by the pentachlorophenyl ester, and the peptide chain was extended from the *C*-terminal residue by mixed anhydride activation of the incoming *N*-protected amino acid. A desired sequence of *N*-protected, *C*-activated molecules was prepared by deprotection of the *N*-protected group, followed by coupling with the next amino acid in the sequence. Coupling was achieved by using mixed anhydride followed by a repetition of this procedure. Satisfactory polymerization was achieved upon removal of the final *N*-protection. One of the main advantages of this approach is the avoidance of the use of alkaline conditions which can lead to racemization and other undesirable side reactions (13). In addition, as the peptide chain is extended from the *C*-terminal residue of the amino acids, the degree of racemization is further limited, since the possibility of racemization of the penultimate residue is nonexistent (14).

This successful approach for the synthesis of simple sequential polypeptides was employed for the synthesis of poly(His-Asp-Ser-Gly). Selection of glycine as the *C*-terminal amino acid component was based upon the observation that it occurs around the active site of the proteolytic enzymes. Also, the incorporation of glycine at the *C*-terminal will minimize the degree of racemization during the polyacylation step.

Scheme I outlines the synthesis of the fully protected tetrapeptide. *N*-Carbobenzoxy(*O*-*tert*-butyl)-Ser was coupled with glycine pentachlorophenyl ester hydrochloride *via* the mixed anhydride method using isobutyl chlorocarbonate and *N*-methylmorpholine as base, to yield dipeptide active ester (I).

This product, as well as subsequent ones, were characterized by the presence of both amide I and II bonds and pentachlorophenyl ester bands in the IR spectrum as indicated in Table I.

Table II—pH Dependence of the Catalytic Coefficient for the Hydrolysis of *p*-Nitrophenylacetate by the Sequential Polypeptide (VIII) [a]

pH	k (liter mole^{-1}min^{-1})
6.4	47
6.9	81
7.4	138
7.9	64
8.4	39

[a] In 0.2 *M* phosphate buffer containing 3.5% dioxane (v/v) at 37°.

Dipeptide (I), was successfully deprotected by catalytic hydrogenolysis to give the dipeptide active ester hydrochloride (II). Using the same procedures, *N*-carbobenzoxy(*O*-*tert*-butyl)-aspartic acid was coupled with dipeptide active ester hydrochloride (II) to give protected tripeptide active ester (III). This tripeptide was deprotected to produce *O*-*tert*-butyl-Asp-(*O*-*tert*-butyl)-Ser-Gly pentachlorophenyl ester hydrochloride (IV). *N*-Carbobenzoxy(imbutyloxycarbonyl)-His was coupled with tripeptide active ester hydrochloride (IV) to give the protected tetrapeptide active ester, *N*-carbobenzoxy (imbutyloxycarbonyl)-His-(*O*-*tert*-butyl)-Asp-(*O*-*tert*-butyl)-Ser-Gly pentachlorophenyl ester (V). This was followed by hydrogenolytic deprotection to give (imbutyloxycarbonyl)-His-(*O*-*tert*-butyl)-Asp-(*O*-*tert*-butyl)-Ser-Gly-pentachlorophenyl ester hydrochloride (VI).

Synthesis of Free Sequential Polymer Poly(His-Asp-Ser-Gly)—Tetrapeptide active ester hydrochloride (VI) was allowed to polymerize in presence of dimethylformamide and triethylamine to yield crude protected polymer (VIII), which was converted to deprotected polymer without further purification as characterization.

A suitable method to remove the *t*-butyl protecting groups was that of a previous study using trifluoroacetic acid (15). Using conditions previously reported (8) produced the unprotected polymer (VIII). The completion of reaction was established by the absence of *t*-butyl group absorption in the IR spectrum as well as titration of free carboxyl groups. After dialysis employing a membrane[6] which retains compounds with molecular weight >5000, a polymeric product containing >15 or 16 repeating units was obtained.

Optical Purity of Sequential Polymer—The optical purity of poly-(His-Asp-Ser-Gly) was calculated by determining the optical activity of the total hydrolysate and comparing it with that of a control. It was found that no significant racemization occurred during the polyacylation reactions. Since all intermediates at each stage were extensively purified prior to use in the synthesis of the tetrapeptide, the optical purity of sequential polymer was relatively high, as expected.

Determination of Catalytic Activity—The catalytic hydrolyses of *p*-nitrophenylacetate by various copolymers have been previously studied (1, 2). Hydrolytic activity of the copolymers was found to depend on pH, temperature, and concentration of the substrate. Stereoselectivity is also a striking characteristic of true enzyme action. A previous study (5) reported the stereoselectivity of synthetic peptide esterase models as a catalyst, suggesting that some of the polyfunctional effects associated with the enzyme active site might indeed be operative in these peptide models. A further study (3) showed that in all cases, first-order kinetics are followed over most of the reaction.

Table II describes the catalytic coefficient for the hydrolysis of *p*-nitrophenylacetate by the sequential polypeptide (VIII) at different pH values at 37°. Highest activity was obtained at pH 7.4. Thus, catalytic activity of the sequential polymer (VIII) was done at pH 7.4, and the results are shown on Table III. While it has a higher catalytic coefficient than histidine, imidazole, or mixture of component amino acids, this sequential polymer failed to give a high catalytic coefficient. This occurred even though the polymer-incorporated amino acids were known to be present in the active sites of various proteolytic enzymes. The low activity may be due to lack of sufficient interactions between the imidazole group of histidine, the hydroxyl group of serine, and the carboxyl group of aspartic acid.

Table III—Catalytic Coefficients (k) for the Hydrolysis of *p*-Nitrophenylacetate by the Sequential Polymer (VIII) and Other Catalysts [a]

Catalyst	Catalytic Coefficient (k) liter moles^{-1}min^{-1}
L-His·HCl	7
Imidazole	48
Sequential Polymer (VIII)	138
L-His+Asp+L-Ser+Gly	18
α-Chymotrypsin	10^4

[a] In 0.2 *M* phosphate buffer, pH 7.4, containing 3.5% dioxane (v/v) at 37°.

The synthesis and catalytic activity of the sequential polypeptide incorporating histidine, aspartic acid, and serine was achieved. The active ester method of polymerization gave excellent yields and moderately high molecular weight polymer with little or no racemization as evidenced by the high degree of optical purity. The successful synthesis of sequential polypeptide H-(His-Asp-Ser-Gly)-OH suggests that this method can be utilized for synthesis of polypeptides containing other functional groups. This sequential polypeptide, containing residues known to be involved in the catalytic activity of chymotrypsin, failed to show significant catalytic activity.

Determination of the absolute value of activity per catalytic center is difficult because the number of active sites depends on the shape of the molecule. Also, this work does not provide any information on the stereospecific hydrolysis of the substrate.

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