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Isolation and Structural Determination of Chromophoric Acyl Peptides from Subtilisin Enzymes*

Harry F. Noller and Sidney A. Bernhard

ABSTRACT: Stable monofurylacryloyl derivatives of two subtilisin enzymes were prepared. Following proteolytic digestion, isolation and structural determination of furylacrylyl peptides showed the acyl group to be present as *O*-furylacryloylserine in the sequence Asp-(NH_2)·Gly·Thr·Ser·Met. The ultraviolet spectra of the denatured furylacrylyl enzymes and furylacryloyl peptides were similar to each other but different from the spectra of the undenatured acyl enzymes. The rates of base-catalyzed deacylation of denatured furylacryloyl enzymes and furylacryloyl peptides were those expected for furylacryloyl esters. Owing to its distinct absorption spectrum, the furylacrylyl group was of great utility both as a label to facilitate isolation of acyl derivatives and as a spectral probe of the chemical nature of acyl linkages.

The enzymes collectively known as "subtilisins" constitute a group of alkaline proteases which can be isolated in crystalline form from various strains of *Bacillus subtilis*. Much of the information which has been obtained regarding their isolation, purification, chemical and physical characterization, and enzymic specificity has been reviewed by Hagihara (1960). Mechanistically, these enzymes have become of interest since the observation that they are inactivated by reaction with 1 mole of DFP per mole of enzyme (Güntelberg and Ottesen, 1954; Ottesen and Schellman, 1957; Matsubara and Nishimura, 1958). More recently, the observation of acyl intermediates during the catalysis of various hydrolytic reactions (Bernhard *et al.*, 1965) similar to those which have been observed for chymotrypsin-catalyzed reactions has served to emphasize the similarities between these and other "serine pro-

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teases." It has been generally implied that the site of reaction of DFP with the enzyme is the site of binding of the specific substrate acyl group during the course of normal enzymatically catalyzed hydrolytic reactions. In the case of chymotrypsin-catalyzed hydrolysis of *p*-nitrophenylacetate, ^{14}C-labeled acetate was found associated with the same serine residue as had been found in the case of ^{32}P-labeled DFP (Oosterbaan and Van Andrichem, 1958).

In previous studies on enzymes which are inactivated by DFP, the site of attachment of the phosphate residue has been shown to be at a single serine oxygen, in the sequence Gly·Asp·Ser·Gly[1] for chymotrypsin (Oosterbaan *et al.*, 1958), trypsin (Dixon *et al.*, 1958), and thrombin (Gladner and Laki, 1958), and in the sequence Gly·Glu·Ser·Ala in aliesterase (Jansz *et al.*, 1959b), pseudocholinesterase (Jansz *et al.*, 1959a), and elastase (Hartley *et al.*, 1959). However, in the case of Novo subtilisin, Sanger and Shaw (1960) have reported the phosphoserine residue from diisopropylphosphoryl–Novo subtilisin in the sequence Thr·Ser·Met·Ala, a sequence quite unlike those mentioned previously. Other bacterial subtilisins contain this same sequence (D. C. Shaw, personal communication, 1963). The latter investigators concluded that the sequence around the reactive serine residue was not responsible for the unusual reactivity toward DFP in these hydrolytic enzymes, but attributed this reactivity to another group within spatial proximity of this serine residue. However, the possibility remained that an aspartic acid or glutamic acid residue would be found adjacent to the threonine residue of this sequence, thus preserving the pattern of carboxylic acid side chain followed by hydroxyalkyl side chain.

Recent developments in the use of specific chromophoric acylating agents in this laboratory (Bernhard *et al.*, 1965) prompted the further investigation of the site of interaction of substrate with enzyme. The use of furyl-2-acryloyl and indole-3-acryloyl acyl groups provides a sensitive probe of the chemical nature of the acyl enzyme linkage, as is reflected in their ultraviolet spectra. In addition, these intense chromophores serve as labels during the course of isolation and purification of acyl enzymes and acyl peptides. For simplicity, the present discussion will be confined to the results obtained with furylacrylate derivatives, although analogous results are obtained with indoleacrylate derivatives. Duplicate studies were performed in most instances with two of the presently available crystalline subtilisins, which will be designated by their trade names, Novo and Nagarse.

[1] Abbreviations used in this work: Ala, alanine; Asp, aspartic acid; Asp(NH_2), asparagine; Glu, glutamic acid; Gly, glycine; Met, methionine; Ser, serine; Thr, threonine; Cbz-, carbobenzyloxy-; SDS, sodium dodecyl sulfate; FDNB, 1-fluoro-2,4-dinitrobenzene; DIP, diisopropylphosphoryl-.

Experimental

Materials

Crystalline bacterial protease Novo (Batch 56) was obtained from Novo Industri, Copenhagen, Denmark. Nagarse-lyophilized crystalline bacterial protease was obtained from Nagase and Co., Ltd., Osaka, Japan. α-Chymotrypsin (thrice crystallized, CDI 6068-9), bovine trypsin (twice crystallized, salt-free, lyophilized, TRL 6227), twice crystallized pepsin (PM 687), carboxypeptidase A (twice crystallized), and leucine aminopeptidase were obtained from Worthington Biochemical Corp., Freehold, N.J. Crystalline porcine trypsin was obtained from the Armour Co., Chicago, Ill. Furyl-2-acrylylimidazole was prepared according to Bernhard *et al.* (1965). Chromatographically pure 1-(+)-serine was obtained from Mann Research Laboratories, New York. *O*-Furylacrylyl-1-(+)-serine was prepared as described later. Ethyl furylacrylate was obtained from K & K Laboratories, Inc., Jamaica, N.Y.

Methods

Ultraviolet Absorption Measurements. Routine optical density measurements were performed with a Beckman DU spectrophotometer, whereas when accurate spectral scans were required a Cary Model 14 recording spectrophotometer was used. The kinetics of reactions were followed spectrophotometrically with a Cary Model 14 or a Beckman Model DB double-beam spectrophotometer with automatic recording attachment. The ultraviolet absorption of the effluent from chromatographic columns was continuously monitored by means of a Vanguard automatic ultraviolet analyzer.

Ultraviolet Spectra of Native and Denatured Acyl Enzymes. To 2.0 ml of a 1.0% solution of enzyme in 0.1 M sodium acetate–acetic acid buffer at *p*H 4.03 was added 10 μl of a 5.0-μmole/ml solution of furylacryloylimidazole in spectrograde acetonitrile. The native acyl enzyme spectrum was determined following addition of 2.0 ml distilled water, and the denatured acyl enzyme spectrum by addition of 2.0 ml of a 0.10 M solution of sodium dodecyl sulfate (SDS).

Base-catalyzed Deacylation of Denatured Acyl Enzymes. To 1.0 ml of a 5.0% solution of enzyme in 0.1 M sodium acetate buffer at *p*H 4.03 was added 30 μl of a 5.0-μmole/ml solution of furylacryloylimidazole in spectrograde acetonitrile. After 1 minute, 4.0 ml of 10.0 M urea was added. The resulting solution was adjusted to an indicated *p*H of 11.83 with KOH and transferred to a quartz spectrophotometer cell. The rate of deacylation was then followed by measurement of the optical density at 320 mμ.

Preparative Acylation Reaction. A typical experiment was performed as follows. Bacterial enzyme (200 mg) was dissolved in 5.0 ml of 0.1 N sodium acetate–acetic acid buffer, *p*H 5.01. Furylacryloylimidazole (10 mg) in 200 μl spectrograde acetonitrile was added to the enzyme solution, followed in 1 minute by 5.0 ml of 5% trichloroacetic acid. The resulting suspension was shaken to ensure complete inactivation of the enzyme. The reaction mixture was centrifuged, the supernatant was

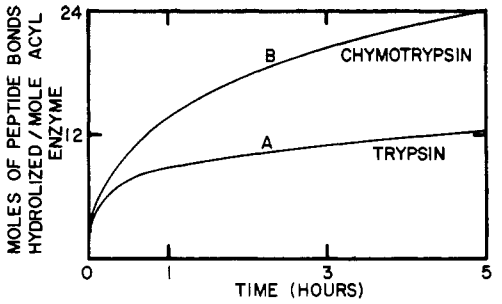


FIGURE 1: Proteolytic digestion of furylacryloyl Novo enzyme by chymotrypsin and trypsin. Denatured furylacryloyl Novo enzyme (200 mg) and 2 mg of chymotrypsin or trypsin were incubated at *p*H 8.0, 25°. The number of peptide bonds split (ordinate) was determined from the quantity of KOH delivered by the *p*H-stat assuming an average *pK* of 7.8 for the dissociation of charged α-amino groups.

decanted, and the precipitated acyl enzyme was washed with three 10-ml portions of anhydrous ether. The precipitate was suspended in 10.0 ml of a solution 0.1 M in KCl and 0.01 M in $CaCl_2$, and adjusted to *p*H 8.0. No residual proteolytic activity was observed after denaturation of either subtilisin by this method, as evidenced by the negligible uptake of alkali in the *p*H-stat at *p*H 8.0 prior to the addition of further enzyme.

Enzymatic Digestion of Acyl Enzyme. The digestion of the acyl enzyme was followed by means of a *p*H-stat consisting of a radiometer TTTl titrator coupled to an Ole Dich recorder (Jacobsen *et al.*, 1957); 2.0 mg of either chymotrypsin or trypsin was added to the stirred suspension of denatured acyl enzyme, and the reaction was allowed to proceed at 25° for 5 hours in the case of chymotryptic digests and up to 24 hours in the case of tryptic digests. Chymotryptic digestion was not complete after 5 hours, but prolonged digestion periods failed to increase acyl peptide yields significantly. Peptic digestion was carried out at *p*H 2.2 and at 37°, using 4.0 mg of pepsin. Peptic digestion was allowed to proceed for 12 hours.

Sephadex G-25 Chromatography. After filtering off of any insoluble material and adjustment of the filtrate to *p*H 7, the enzymatic digest was placed directly on a 2- × 215-cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden), and eluted with distilled water. Appropriate fractions as judged from the ultraviolet absorption of the effluent, were pooled, evaporated to dryness under reduced pressure, and stored at −13° until needed.

Dowex 1-X2 Chromatography. Bio-Rad AG 1-X2 anion exchange resin (200–400 mesh) was prepared according to the method of Rudloff and Braunitzer (1961) and equilibrated with 0.2 N ammonium acetate at *p*H 6.9 prior to use. Samples to be chromatographed were dissolved in 2.0 ml of the latter solvent for application to the 2- × 50-cm column. Gradient elution was employed in most instances, utilizing a 500-ml mixing chamber (containing 325 ml of the initial buffer) into which the second buffer could flow from a separatory funnel. Each subsequent gradient was initiated by removing the remaining buffer from the reservoir and introducing the next buffer, leaving the existing solution in the mixing chamber. Fractions were pooled as described, and stored at 4° or −13° until needed.

Desalting Peptide Fractions. Desalting was accomplished by means of a short column (2 × 8 cm) of AG 1-X2, equilibrated at *p*H 7 with 0.2 N ammonium acetate, which was washed with 50 ml of distilled water prior to application of the sample. The fraction to be desalted (*ca.* 200 ml) was adjusted to *p*H 7 and passed through the column, whereupon the peptide was adsorbed to the column. The remaining salt was washed from the column with several 50-ml portions of distilled water, after which the peptide was eluted with about 15 ml of 0.2 N acetic acid. The peptide solution thus obtained was finally concentrated to dryness, and the traces of acetic acid were removed under reduced pressure over NaOH by redissolving in 5 ml distilled water and evaporation as previously for three successive times.

Amino Acid Analysis. Amino acid analyses were performed on a Spinco Model 120 or Model 120B amino acid analyzer according to the method of Spackman *et al.* (1958). Peptides were hydrolyzed under reflux with twice-distilled constant-boiling HCl for 20 hours. Samples were evaporated to dryness under reduced pressure over NaOH, twice redissolved in distilled water followed by evaporation as above, and finally taken up in 1–2 ml of 0.2 N sodium citrate buffer (*p*H 2.2) for application to the analyzer column. A quantity of 0.1 to 1.0 μmole of material was used for each analysis.

Carboxypeptidase A Digestion. Carboxypeptidase solutions were prepared utilizing the extinction (at 280 mμ) reported by Hirs *et al.* (1960). In a typical carboxypeptidase degradation, 1 μmole of peptide was dissolved in 1 ml of 0.2 M Tris-HCl buffer, *p*H 8.0. At time zero, 100 μl of a concentrated carboxypeptidase solution was added with thorough mixing to give a final concentration of 0.025 mg carboxypeptidase A per ml. The reaction was allowed to proceed at 25°. Aliquots (200 μl) of the reaction mixture were removed at specified time intervals, immediately pipetted into 1.0 ml of 0.1 N HCl, and frozen until ready for use. Controls, which contained all components of the reaction mixture except peptide, were run simultaneously.

Leucine Aminopeptidase Digestion. To 1.0 μmole of peptide in 1.0 ml of distilled water were added 50 μl of 0.2 M $MnCl_2$ and 50 μl of 1 M Tris-HCl buffer, *p*H 8.5, and the mixture was incubated at 40.0°. At time zero, 20 μl of a 0.1-mg/ml or 5.0-mg/ml solution of leucine aminopeptidase in 0.01 M $MnCl_2$ was added. Aliquots (200 μl) were removed at specified time intervals and pipetted immediately into 1.0 ml of 0.1 N HCl, and frozen until ready for use.

Reaction of Peptides with 1-Fluoro-2,4-dinitrobenzene (FDNB). A modification of the method of Sanger and Thompson (1953) was used as follows: To 0.4 μmole of peptide in 0.4 ml of 1% triethylamine were added 2.0

μmoles of FDNB (Matheson, Coleman and Bell, mp 23–25°) in 0.8 ml absolute ethanol. The reaction mixture was incubated in a glass-stoppered test tube at 40° for 4 hours. It was then evaporated to dryness under reduced pressure, redissolved in 1 ml of 0.01 N HCl, and again taken to dryness. The cold finger treatment of Mills (1952) was used for sublimation of dinitrophenol, and the sample was then divided for 5- and 16-hour reflux periods in constant-boiling HCl. The respective hydrolysates were diluted by addition of 4 volumes of distilled water, and extracted with ether. The aqueous phase was prepared for amino acid analysis as described, and the DNP-amino acids were chromatographed on paper in the *tert*-amyl alcohol–potassium phthalate system of Blackburn and Lowther (1951), along with authentic samples of DNP-amino acids for comparison and identification. If further doubt existed as to the identity of a DNP derivative, the derivative was eluted from the paper after chromatography in the first solvent, and rechromatographed in 1.5 M phosphate buffer, *p*H 6.0.

Preparation of O-Furylacryloyl-L-serine. To 4.0 ml of a solution containing 2.5 M L-serine and 2.0 M imidazole at *p*H 7 was added dropwise 20 mg of furylacryloylimidazole in 1.0 ml acetonitrile. After the solution had been stirred for 1 hour, isopropyl alcohol was added until no further precipitation occurred. The precipitate, which was largely unreacted serine, was filtered off, and the filtrate was stored at −13°. The filtrate contained approximately 2.6 μmoles of *O*-furylacryloylserine per ml, small aliquots of which were diluted 100-fold for spectral purposes. The spectrum of the product had an ultraviolet absorption spectrum at 308 mμ, corresponding to that of a substituted furylacryloyl ester (Bernhard *et al.*, 1965).

Base-catalyzed Deacylation of Furylacryloyl Derivatives. The rates of base-catalyzed deacylation of furylacryloyl esters, amino acids, and peptides were measured spectrophotometrically. A solution of the furylacryloyl derivative containing approximately 0.05 μmole/ml of distilled water or 8 M urea was adjusted to *p*H 11.80–11.85 (uncorrected for urea concentration) with KOH, and the kinetics of deacylation was followed spectrophotometrically at 320 mμ (Bernhard *et al.*, 1965).

Results

Formation and Stability of Acyl Enzyme. At *p*H 5, the formation of acyl enzyme is extremely rapid and, under the conditions used for the preparative experiments, a 30-second reaction period was sufficient to effectively convert all the enzyme to furylacryloyl enzyme. This conversion was accompanied by a shift in the ultraviolet absorption maximum of the chromophore from 340 to 320 mμ, and a loss of activity of the enzyme. The spectrum of the resulting acyl enzyme is shown in Figure 5. Deacylation, on the other hand, takes place at a much slower rate at this *p*H, allowing the formation of a metastable intermediate. Precipitation of furylacryloyl enzyme with trichloroacetic acid resulted in an irreversible denaturation (no detectable proteolytic activity returned upon bringing the denatured acyl enzyme to *p*H 8).

After acylation occurred, deacylation could be brought about by raising the *p*H to neutrality, releasing the furylacrylate anion, which is clearly distinguishable by its absorption spectra at acidic and basic *p*H (Bernhard *et al.*, 1965). The stoichiometry of the acylation reaction could then be calculated from the extinction coefficient of the furylacrylate anion, and estimates of the molecular weight and purity of the enzyme. An analogous procedure, in which the anion was removed by saponification at *p*H 12, was applicable for the denatured acyl enzymes and acyl peptides. In all cases, it was clear that no more than one acyl group per molecular weight 30,000–40,000 had been bound to the enzyme. In the peptides isolated, only one furylacryloyl group was attached.

Denaturation of the furylacryloyl enzyme with SDS frequently did not completely irreversibly inactivate the enzyme, since proteolytic activity was observed in many cases after removal of the SDS. For this reason, trichloroacetic acid precipitation was the method of choice for denaturation of the acyl enzyme, without concomitant deacylation, prior to proteolytic digestion.

Enzymatic Digestion of the Acyl Enzyme. Although trypsin cleaves a large number of the acyl enzyme peptide bonds (Figure 1A), an insoluble "core" remains. Less than 5% of the furylacryloyl constituent is found in soluble peptides. Attempts to dissolve the core material in various solvents were unsuccessful.

Chymotrypsin digestion is more rapid, and more bonds are susceptible to cleavage by chymotrypsin than by trypsin (Figure 1B). Relatively little insoluble material was encountered at the termination of the chymotryptic and peptic digests. Approximately 40% of the theoretical amount of acyl peptide was usually obtained, assuming a molecular weight of 30,000 for the enzyme (Hagihara, 1960), and assuming furthermore that the commercial enzyme preparation was 100% pure.

Chromatography of Chymotryptic and Peptic Digests on Sephadex G-25. Chromatography of the chymotryptic digest on Sephadex G-25 resolved the acylated fragments into five main peaks (Figure 2A). Peak D-chy (Figure 2) contained most of the acyl peptide material, and the ultraviolet spectra of fractions in this region showed proportionally less ultraviolet-absorbing (280 mμ) nonacylated material than the other peaks. From the properties of Sephadex G-25 (Flodin, 1962), it was assumed that peptides which were eluted in relatively late fractions would be the smaller ones, and hence of a more convenient size for initial structural determinations. Peak D-chy was therefore subjected to further purification by ion-exchange chromatography on Dowex 1-X2 (*vide infra*).

Chromatography of the peptic digest on Sephadex resulted in resolution of the mixture into four ultraviolet-absorbing peaks (Figure 2B). The elution pattern of peptic peptides on Sephadex indicates that the products of peptic digestion were, on the average, smaller than the chymotryptic peptides. Most of the furyl-

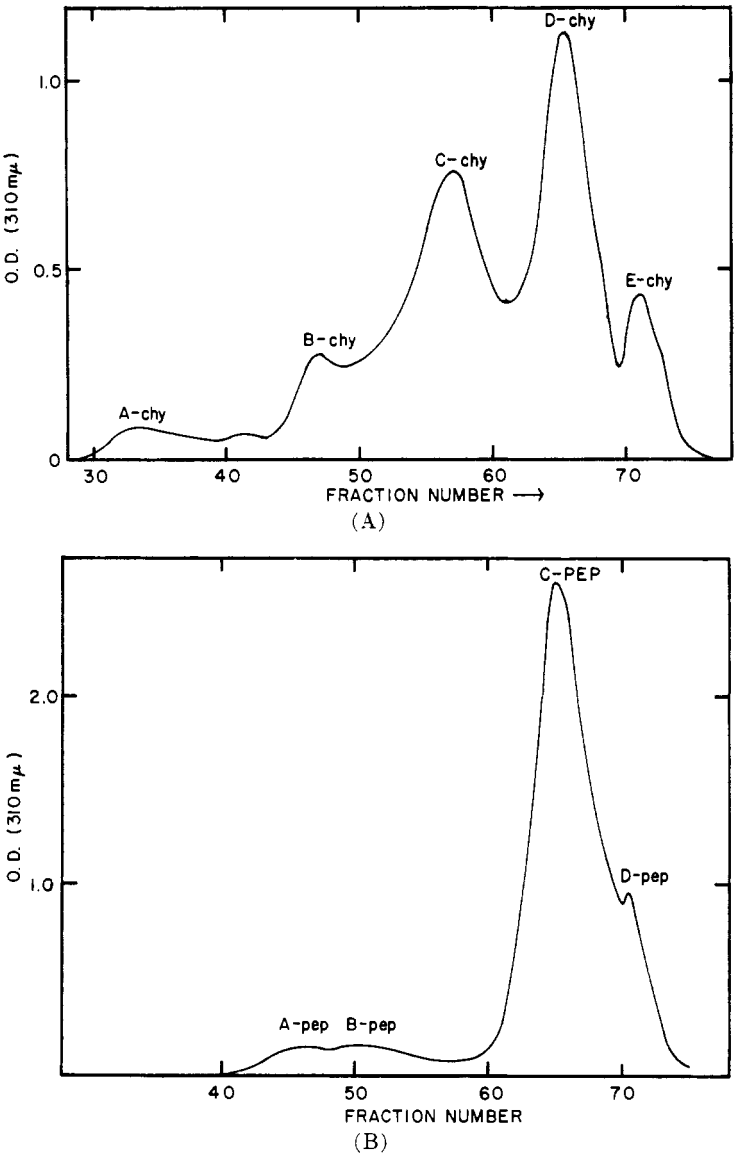


FIGURE 2: Chromatography of proteolytic digests of furylacryloyl Novo enzyme on Sephadex G-25. Fraction volumes were 7.4 ml. Chromatography was carried out at 25°. (A) Chymotryptic digest; (B) peptic digest.

acryloyl-containing peptides were eluted in a large peak, C-pep, with an elution position corresponding to that of D-chy. The fractions comprising peak C-pep were pooled, further purified (*vide infra*), and employed in structural determination.

Chromatography of Sephadex Fractions on Dowex 1-X2. Figure 3A shows the chromatogram resulting from gradient elution (from *p*H 6.9 to 4.5 with 0.2 N ammonium acetate buffers) of the acyl peptide material from peak D-chy. It is seen that two peptide components were eluted under these conditions. The major component, D-chy-II, was isolated in quantities ranging from 1 to 1.5 μmoles (*ca.* 15–25% overall recovery based on the foregoing assumptions). The small peak appearing at fraction 21 did not exhibit the furylacryloyl spectrum and was not further investigated. No additional acyl peptide material could be eluted from the column, even when the gradient was extended to 50% glacial acetic acid, with the furylacryloyl enzymes. When indoleacryloyl derivatives were investigated, the acyl peptides adsorbed to the resin so strongly that they could be eluted only with 10–50% acetic acid. Six indoleacryloyl derivatives could be isolated under these conditions; however, the yields of each were comparatively small and therefore investigation of these peptides was postponed in favor of the more conveniently obtainable furylacryloyl peptides.

Figure 3B shows the Dowex 1-X2 chromatography of Sephadex fraction C-pep, using the same gradient employed for D-chy. The two furylacryloyl peptides, C-pep-I and C-pep-II, were isolated for further investigation. The ultraviolet-absorbing material preceding

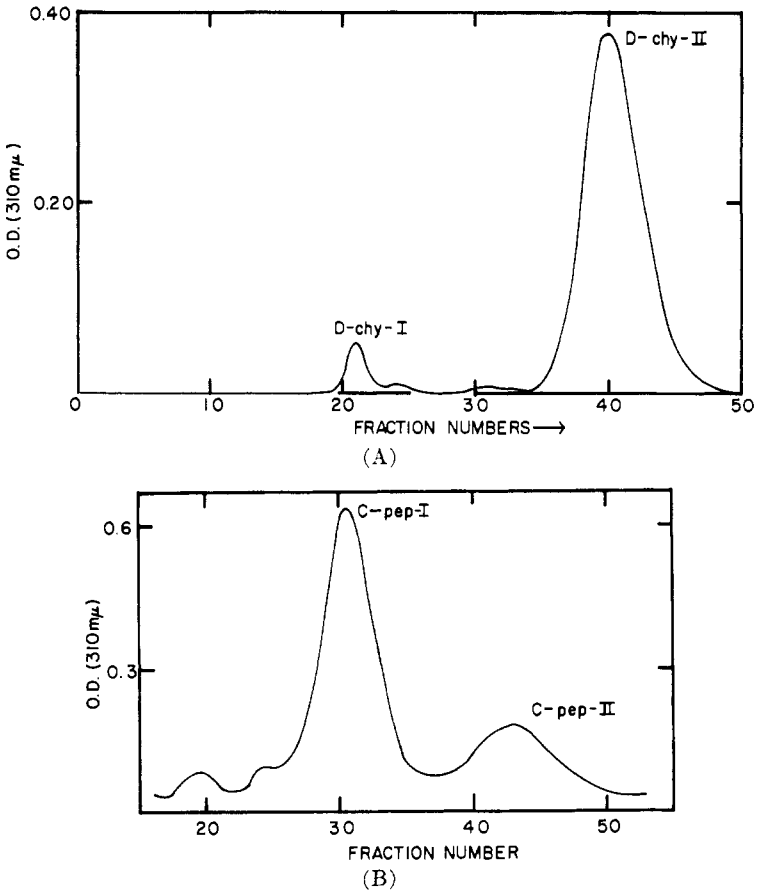


FIGURE 3: Chromatography of (A) fraction D-chy and (B) fraction C-pep on Dowex 1-X2. A *p*H gradient from *p*H 7 to 4.5 was employed in both instances, beginning at fraction 1. Chromatography was carried out at 25°; volume of each fraction was 16.4 ml.

C-pep-I did not exhibit the furylacryloyl spectrum and was not investigated further.

Amino Acid Compositions of Furylacryloyl Peptides. The amino acid analyses of D-chy-II and C-pep-I indicated only trace contamination from other peptides and amino acids. Peptide concentration prior to analysis could be determined by measuring the furylacryloyl absorption using a molar extinction coefficient of $\epsilon^{310} = 2.1 \times 10^4$. In expressing amino acid compositions in molar ratios, serine was arbitrarily set equal to 1.00. The amino acid composition of D-chy-II from Table I is (Asp, Thr, Ser, Gly, Met) and C-pep-I is (Asp, Thr, Ser, Gly). Prior use of ammonium acetate buffers made ammonia determinations unfeasible, and hence these analyses did not distinguish between aspartic acid and asparagine. Trace contaminants amounted to less than 0.05 residue.

Degradation of D-chy-II with Carboxypeptidase A. Table II shows the release of amino acids from D-chy-II by carboxypeptidase A after varying lengths of time. Only trace amounts (less than 0.05 residue) of amino acids other than methionine are released, even after prolonged digestion. These trace amounts may be attributed to contaminating peptides or nonenzymatic material in the enzyme preparation, which is slowly degraded by carboxypeptidase. Controls, containing all reaction components except peptide, also showed slow release of trace quantities of amino acids. Although com-

TABLE I: Amino Acid Compositions of Furylacryloyl Peptides.

	D-chy-II		C-pep-I
	Novo	Nagarse	Novo
Aspartic acid	1.11	1.14	1.10
Threonine[a]	0.98	1.09	0.98
Serine[a,b]	1.00	1.00	1.00
Glycine	0.94	1.08	1.06
Methionine[a]	0.88	0.91	

[a] No correction was made for loss of serine, threonine, or methionine during the course of acid hydrolysis. [b] The value for serine was arbitrarily set equal to 1.00. The furylacryloyl/serine ratio was unity within experimental error in all peptides.

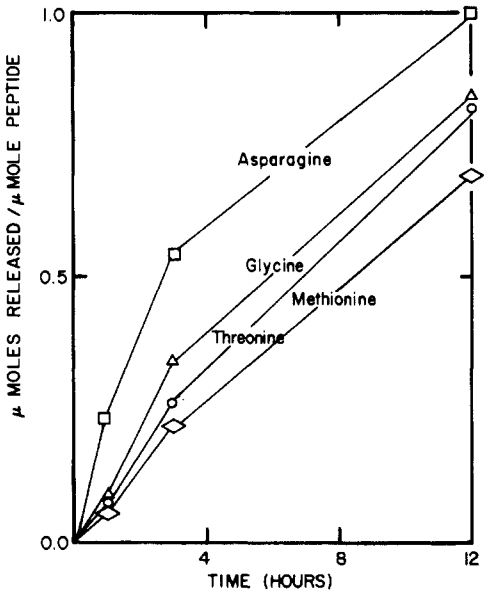


FIGURE 4: Release of amino acids from D-chy-II during leucine aminopeptidase degradation. The 12-hour asparagine value was not corrected for the slow release of serine owing to alkaline hydrolysis of *O*-furylacryloylserine in the digestion mixture. Other values were based on a 12-hour asparagine value of 1.00.

TABLE II: Release of Amino Acids from Peptide D-chy-II by Carboxypeptidase A.

Time	Moles Released/Mole of Peptide				
(min)	Asp	Thr	Ser	Gly	Met
5		tr	tr		0.68
20			tr		0.90
1440	tr	tr	tr		1.00

plete release of methionine is rapidly attained, it is evident that the action of carboxypeptidase on D-chy-II is blocked after the release of this single residue.

Degradation of D-chy-II with Leucine Aminopeptidase. Release of amino acids from the N-terminal end of D-chy-II by leucine aminopeptidase is shown in Figure 4. It is evident from this procedure that the aspartic acid residue isolated by total acid hydrolysis is, in actuality, asparagine in the enzyme. The peptide was totally degraded by aminopeptidase. The order of release of the amino acid residues is $AspNH_2$, Gly, Thr, Met. Almost simultaneously with the release of methionine from the peptide, a "new" amino acid can be chromatographically isolated on a 150-cm column in the amino acid analyzer (having an elution position 203 ml after the buffer change in a standard 50° run). The tentative identification of this component as *O*-furylacryloylserine was subsequently confirmed by repeating the analysis procedure with an authentic sample.

N-Terminal Analysis by the FDNB Method. The FDNB reaction was performed on both D-chy-II and C-pep-I. Both 4- and 16-hour hydrolyses were performed on C-pep-I. The amino acid compositions of the residual peptides, after extraction of DNP-amino acids, are given in Table III. Loss of aspartic acid is clearly in evidence in all three determinations. Paper chromatography of

TABLE III: Residual Amino Acid Composition after Reaction with FDNB.

	D-chy-II	C-pep-I	
	16 hours	4 hours	16 hours
Asp	0.23	0.29	0.34
Thr	0.98	0.90	0.93
Ser	1.00	1.00	1.00
Gly	1.02	0.96	1.18
Met	1.01		

the DNP-amino acids revealed a spot corresponding to DNP-aspartic acid in the 16-hour hydrolysate, but the 4-hour hydrolysate contained an additional spot which corresponded to DNP-glycine, while the DNP-aspartic acid spot was missing. Since the amino acid compositions of the hydrolysates do not indicate the loss of glycine in the 4-hour hydrolysis, the spot identified as DNP-glycine must be an artifact. The N-terminal amino acid residue is therefore asparagine.

Base-catalyzed Hydrolysis of Furylacryloyl Derivatives. The base-catalyzed hydrolysis of the furylacryloyl peptide C-pep-I was observed to be first order with respect to peptide concentration, as was also observed for the analogous hydrolysis of ethyl furylacrylate, *O*-furylacryloyl-L-serine, and urea-denatured furylacryloyl Novo enzyme. The pseudo-first-order saponification rate constants for these reactions are given in Table IV. All

TABLE IV: Base-catalyzed Hydrolysis of Furylacryloyl Derivatives at *p*H 11.8,[a] 25°, in 8 M Urea.

	$k \times 10^2$ (min^{-1})
C-pep-I	1.73
Furylacryloyl Novo (urea-denatured)	1.82
O-Furylacryloylserine	1.22
Ethyl furylacrylate	0.37
Furylacryloyl Novo (native)[b]	410

[a] The first four rates are first order in $[OH^-]$ in this *p*H range. [b] Not in urea, but in 0.10 M pyrophosphate buffers, *p*H 8.8–10.0. The rate is virtually *p*H independent over this *p*H range.

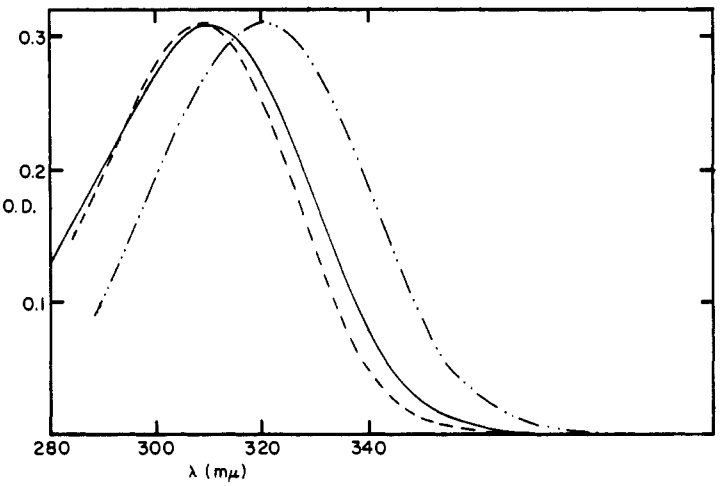


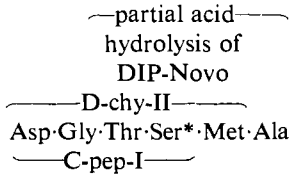
FIGURE 5: Ultraviolet spectra of furylacryloyl derivatives. (———), C-pep-I; (– – – –), SDS-denatured fu ylacryloyl Novo enzyme; (— · · —), native furylacryloyl Novo enzyme. Peptide spectrum was measured versus distilled water. Enzyme spectra were run as difference spectra versus identical samples which lacked only the furylacrylate component.

rates are compatible with the rates expected for furylacryloyl esters.

Ultraviolet Spectra of Furylacryloyl Derivatives. The ultraviolet absorption spectra of the furylacryloyl peptide C-pep-I, native furylacrylyl Novo enzyme, and SDS-denatured furylacrylyl Novo enzyme are shown in Figure 5. The spectrum of the furylacryloyl peptide is similar to both the ethyl ester (Bernhard *et al.*, 1965) and denatured enzyme, but quite unlike that of the native enzyme, which is displaced toward the red by about 10 mμ.

Discussion

N-Terminal degradation with leucine aminopeptidase, the results of which are shown in Figure 4, indicate that the amino acid sequence of D-chy-II is Asp(NH_2)·Gly·Thr·Ser·Met.[2] The C-terminal position of methionine is confirmed by the results of carboxypeptidase A hydrolysis, which releases only methionine. N-Terminal asparagine is confirmed by loss of aspartic acid from the acid hydrolysate after reaction of both D-chy-II and C-pep-I with FDNB. The point of attachment of furylacrylate is established as the oxygen of serine by the absence of serine in the total leucine aminopeptidase hydrolysate of D-chy-II, and by comparison of the new peak appearing upon amino acid analysis of the above with a known sample of *O*-furylacrylyl serine. The Thr·Ser·Met portion of the sequence contained in the peptides obtained by acid hydrolysis of DIP-Novo by Sanger and Shaw (1960) confirms the central portion of the sequence presented here (since both inhibitors are observed to inactivate the enzyme stoichiometrically by reaction with a single serine residue per mole of enzyme). The total known sequence surrounding the reactive serine residue in Novo subtilisin is thus:



The work to date on the Nagarse enzyme suggests that the same sequence exists in this region for both enzymes, although the C-terminal alanine has not yet been demonstrated in the Nagarse enzyme.

Absence of a carboxylic side chain in the proximity of the serine residue clearly distinguishes these subtilisin enzymes from other "serine proteases." It is clear, as suggested by Sanger and Shaw (1960), that a carboxyl group adjacent to the serine in sequence is not necessary for the catalytic activity exhibited by these enzymes, although one cannot exclude the possibility that a sequentially distant aspartic or glutamic side chain may be spatially close to the reactive serine side chain in the folded native enzyme.

Another possibility which has *not* been ruled out is that the catalytic site of acylation may be different from the "isolated" site of acylation. One cannot exclude an intramolecular transfer from a more reactive acylated site to a relatively more stable serine ester derivative upon denaturation of the enzyme prior to degradation. For example, the tetrapeptide carbobenzyloxy·Thr·Ser·Met·Ala·Me reacts very rapidly with acylimidazoles to yield the corresponding *O*-acylserine ester (Bernhard *et al.*, 1964). Presumably, by analogy, the serine hydroxyl could react still more rapidly with an intramo-

[2] The sequence, Gly·AspNH_2·Thr·(*O*-DIP)Ser·Met·Ala·Ser·Pro·His, has been recently reported for DIP-subtilisin (R. A. Oosterbaan, Abstracts of the VI International Congress of Biochemistry, N.Y., July 26–Aug. 1, 1964). We are in disagreement on the order of the N-terminal dipeptide in this fragment.

lecular *N*-acylimidazolylhistidine residue in spatial proximity. The spectrum of native furylacryloyl Novo enzyme is quite different from the spectrum of denatured furylacryloyl Novo enzyme, whereas the spectra of denatured acyl enzyme, acyl peptide, and acyl ester are all quite similar (Figure 5). The spectra of furylacryloyl-X derivatives are quite sensitive to the chemical nature of the derivative (X) in question. Furylacryloyl esters, amides, imidazoles, and anions differ from each other by 10–50 mμ in their λ_{max} whereas solvent and hydrogen bonding effects can bring about only small shifts in λ_{max} toward longer wavelengths (Bernhard *et al.*, 1965). In addition, rates of base-catalyzed hydrolysis of furylacryloyl peptide, denatured furylacryloyl enzyme, and ethyl furylacrylate are also similar to each other, yet quite different from that of native furylacryloyl enzyme (Table IV).

The results presented herein emphasize the utility of chromophoric acyl groups such as furylacrylate and indoleacrylate, both as labels and as probes of the chemical environment of the active centers of proteolytic and esteratic enzymes.

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