STUDIES ON THE SPECIFICITY OF BACILLUS SUBTILIS NEUTRAL PROTEASE WITH INSULIN B-CHAIN

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Received May 12, 1967

The substrate specificity of a purified neutral protease obtained from Bacillus subtilis strain AM fermentation beers has been studied using the B-chain of bovine insulin. Previous studies with synthetic substrates indicated that the enzyme cleaved peptide bonds in which the amino group was donated by leucine, phenylalanine or valine (Feder, 1966). It was found that the four bonds adjacent to leucine and the two bonds involving phenylalanine were hydrolyzed in the presence of the enzyme.

Materials and Methods

The neutral protease used in these studies was an ammonium sulfate fractionated preparation which had been treated with DEAE and then purified by column chromatography on hydroxylapatite. The enzyme, though homogeneous by ultracentrifugation, disc gel electrophoresis and gel exclusion chromatography, was treated with 10^{-3} M diisopropyl fluorophosphate to inactivate any trace amounts of the alkaline protease which might be present The insulin (bovine) B-chain oxidized, carboxymethylated B-chain (reduced) and carboxymethylated A-chain (reduced) were purchased from Mann Research Laboratories, Inc. and used without further purification.

About 70 mg of each of the peptides were dissolved in 4.0 ml of 0.2 M phosphate, pH 8.4 and 0.5 ml of this was used as a substrate control. To the remaining 3.5 ml was added 0.1 ml of a 1% neutral protease solution.

The reaction was carried out for 5.5 hours at 25° C, and then frozen and lyophilized. An enzyme control consisting of 3.5 ml of the phosphate solution and 0.1 ml of 1% neutral protease was also carried along in a similar fashion. The lyophilized material was dissolved in about 0.7 ml of 0.053 N NH_4OH and spotted on Whatmann No. 3MM filter paper sheets (46 x 57 cm). The total material was divided up such that about 5-7 mg were spotted on each of two sheets to be sprayed with ninhydrin for locating the peptides separated. The remaining material was spotted on three sheets. Peptide maps were then prepared by the method of Katz, Dreyer and Anfinsen (1959).

Descending chromatograms were run for 16 hours in a solvent system consisting of n-butanol-acetic acid-water (4:1:5). The papers were air dried rotated 90 degrees and high voltage electrophoresis carried out using a 6ME Model DW Electrophorator in a pyridine-acetic acid-water (1:10:289) buffer, pH 3.7. The electrophoresis was carried out for one hour at 2000 volts and a current of 100-135 milliamps.

After electrophoresis the papers were dried with warm air and the two sheets were stained with a 0.2% ninhydrin spray (Block, Durrem and Zweig, 1958) The location of the spots was made with the stained sheets and the peptides were eluted with 6N constant boiling HCl and directly hydrolyzed for 24 hours at 110° C in sealed, evacuated tubes. The amino acid composition of the eluted peptides was determined by a Beckman model 120B amino acid analyzer with an accelerated run (4 hours) and the peak areas were measured by a digital integrator.

The identity of the peptides with respect to its position in the chain was quite evident from the amino acid composition of the spots.

Results and Discussion

Figure 1 shows the peptide map for the neutral protease catalyzed hydrolysis of the carboxymethylated reduced B-chain of insulin. Some of the eluted spots contained more than one peptide, but they could be readily identified from the amino acid composition. Table 1 shows the amino acid

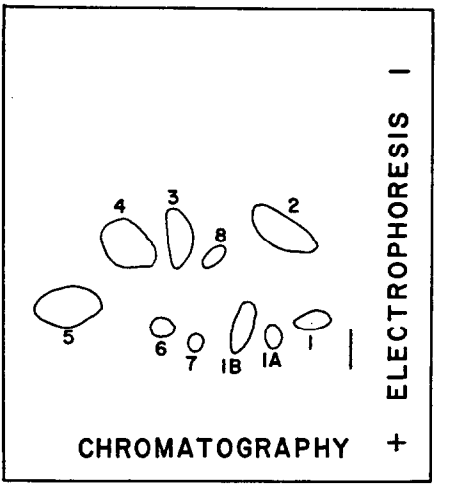

Figure 1. Peptide map of the neutral protease catalyzed hydrolysis of insulin B-chain oxidized. The chromatographic and electrophoretic condition are the same as described in text.

composition of those spots identified and a comparison of the number of residues found and the theoretical values for the composition determined. The amino acid composition of those spots which contained two peptides yielded the percent of each peptide. Spot number one, for example, contained 0.226 micromoles of serine and 0.246 micromoles of histidine, both of which are found in peptide Leu_6-His_{10} but not in the peptide Leu_{17}-Gly_{23}. On the other hand, 0.154 micromoles of valine and 0.153 micromoles of glutamate were found which are part of the peptide Leu_{17}-Gly_{23}. It would therefore appear that the spot consisted of about 0.226 micromoles of peptide Leu_6-His_{10} and 0.154 micromoles of peptide Leu_{17}-Gly_{23}. The theoretical number of residues for the various amino acids was then calculated for this mixture and compared to the results obtained as shown in Table 1. Table 2 shows the results obtained for the similar digestion of the oxidized B-chain of insulin. Both digestions yielded essentially the same peptides. Examination of the peptides obtained indicate that the neutral protease cleaved the peptide bonds between His_5-Leu, His_{10}-Leu, Ala_{14}-Leu, Tyr_{16}-Leu, Gly_{23}-Phe and Phe_{24}-Phe. No cleavage was observed of a peptide bond in which the

Table 1

Amino Acid Analyses of Peptides from Neutral Protease Catalyzed Hydrolysis of Carboxymethylated B-Chain Insulin

Spot	1		1a		2		3		4		5	
Amino Acid	F*	T**	F	T	F	T	F	T	F	T	F	T
Phe					1.13	1.00	3.40	3.26	1.85	2.00	1.54	1.52
Val	1.01	1.00	1.00	1.00	1.00	1.00	0.98	1.00			2.29	2.52
Asp					1.00	1.00	.89	1.0				
His	1.61	1.46			0.89	1.00	0.78	1.00				
Leu	2.37	2.46	1.25	1.00							2.91	2.52
Cys		2.46		1.00								1.52
Gly	3.40	3.46	2.19	2.00							3.54	3.04
Ser	1.46	1.46										
Glu	1.00	1.00	1.08	1.00	1.04	1.00	1.00	1.00			2.46	2.52
Ala							2.27	2.26	1.15	1.00	1.00	1.00
Tyr							1.98	2.26	1.00	1.00		
Arg	0.79	1.00	0.73	1.00							1.14	1.52
Thr							2.16	2.26	0.91	1.00		
Pro							2.11	2.26	0.77	1.00		
Lys							2.20	2.26	1.00	1.00		
Assumed	1 Glu		1 Val		1 Asp		1 Glu		1 Lys		1 Ala	
Peptide	Leu_6-His_{10} 59%		Leu_{17}-Gly_{23}		H_2N-Phe_1-His_5		Phe_{25}-Ala_{30}-COOH 69%		Phe_{24}-Ala-COOH		Leu_{11}-Ala_{14} 40%	
	Leu_{17}-Gly_{23} 41%						H_2N-Phe_1-His_5 31%				Leu_{17}-Phe_{24} 60%	

* Found

** Theoretical for peptides indicated.

Table 2

Amino Acid Analyses of Peptides from Neutral Protease Catalyzed Hydrolysis of Oxidized B-Chain Insulin

Spot	2		4		7		9	
Amino Acid	Found	Theoretical	Found	Theoretical	Found	Theoretical	Found	Theoretical
Phe	1.110	1.39					1.79	1.63
Val	0.42	0.39	0.83	1.00	1.11	1.00	2.62	2.63
Asp	0.42	0.39						
His	0.37	0.39			1.34	1.00	1.15	1.00
Leu			1.96	2.00	2.00	2.00	3.97	3.63
Cys						2.00		
Gly					3.38	3.00	4.36	4.26
Ser					1.18	1.00	0.92	1.00
Glu	0.42	0.39	0.83	1.00	1.14	1.00	2.67	2.63
Ala	0.77	1.00	1.00	1.00			0.95	1.00
Tyr	1.03	1.00	0.95	1.00				
Arg					0.98	1.00	1.46	1.63
Thr	1.08	1.00						
Pro	1.29	1.00						
Lys	1.10	1.00						
Assumed	1 Tyr		1 Ala		2 Leu		1 Ser or 1 Ala	
Peptide	H_2N-Phe_1-His_5 28% Phe_{25}-Ala_{30}-COOH 72%		Leu_{11}-Val-Tyr_{16}		Leu_6-Cys-Ala-Ser-His_{10} 28% Leu_{17}-Val-Cys-Gly-Glu-Arg-Gly_{23} 72%		Leu_6-Ala_{14} 38% Leu_{17}-Phe_{24} 62%	

amino group was donated by valine. The synthetic substrate studies, however, indicated this to be a susceptible bond. Since the bonds involving leucine are much more readily cleaved one would first obtain those peptides, i.e. Leu_{11}-Val-Glu-Ala, Leu_{17}-Val_{18}-Phe_{24} and Leu_{17}-Val_{18}-Gly_{23}. In each case the susceptible valine bond would be adjacent to a free amino group. These bonds were previously shown not to be cleaved by the neutral protease. Only one peptide was identified in the hydrolysis of the carboxymethylated A-chain of insulin. This corresponded to Leu_{13}-Tyr-Glu and indicating that the susceptible bonds were Ser_{12}-Leu and Glu_{15}-Leu.

The results presented also agree with earlier observations that only leucine and phenylalanine were found to be the amino terminal amino acids obtained upon treatment of the products of the neutral protease catalyzed hydrolysis of these peptides with fluorodinitrobenzene (Feder, 1966). The specificity requirements of neutral protease as approximated from the studies of simple dipeptide substrates appear to also hold true for the enzyme catalyzed hydrolysis of the A and B-chains of insulin.

The Bacillus subtilis neutral protease displays a substrate specificity which is quite similar to that found for thermolysin by Matsubara and coworkers (1965, 1966).

Acknowledgement

The authors gratefully acknowledge the technical assistance of Mr. Richard A. Martin who ran the amino acid analyses.

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