Kinin Formation and Inactivation by Alcalase, a Proteolytic Enzyme[1] (34237)

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Alcalase, an enzyme preparation manufactured by the submerged fermentation of a species of the genus bacillus hydrolyzes most common proteins. The enzyme, being stable over a wide pH range and active at temperatures up to 65–70° is widely used in enzymatic detergents. In high concentrations, as occasionally encountered during production, the enzyme may cause irritation of skin and mucous membranes (1). The nature of this inflammatory reaction is not fully understood. The possibility of plasma kinin participation has been considered. Other proteolytic enzymes, such as trypsin, release bradykinin and related peptides (2, 3). The present experiments were undertaken to determine whether Alcalase incubated *in vitro* with human plasma leads to kinin formation. Alcalase was also investigated for kinin-destroying activity.

Material and Methods. Citrated human plasma (4.5 ml) was preincubated for 10 min with 0.6 ml of 2.62×10^{-2} *M* EDTA and 1.25 ml of 0.5 *M* phosphate buffer, pH 6. EDTA is a potent inhibitor of plasma kininase (4), and also the mild acid pH significantly reduces plasma kininase activity (5). The pH of the buffered plasma is 6.6. Alcalase in 0.25 ml of 0.5 *M* phosphate buffer was then added and incubated for 120 or 240 min at 37°. The final enzyme concentration was 0.2 mg/ml. Control incubations of equal amounts of Alcalase, EDTA, and phosphate buffer in 4.5 ml of physiological saline were also performed. Samples from the incubations were withdrawn at various intervals and added to 3 vol of boiling absolute alcohol, boiled for 4 min, prepared for assay, and assayed on the estrous rat uterus according to a procedure previously described (6).

To identify the material formed during incubation as a kinin peptide, samples from the incubations, equivalent to approximately 250 mμg of bradykinin, were incubated with 100 μg of chymotrypsin in 1 ml of 0.5 *M* phosphate buffer, pH 7.4. Control samples from the same incubations were incubated with buffer alone. Furthermore, parallel incubations of citrated human plasma, EDTA, and phosphate buffer, pH 6, were made with and without Trasylol, 2000 units/ml. Trasylol is a commercially available kallikrein inhibitor from beef organs (mol wt of 6700) consisting of 58 amino acids in a well defined sequence (7).

Investigations for kinin-destroying activity were performed by adding 2250 mμg of synthetic bradykinin (Sandoz) to Alcalase in 4.5 ml of saline; 1.50 ml of phosphate buffer, pH 6.6; and 0.6 ml of 2.62×10^{-2} *M* EDTA solution. The final enzyme concentration was 0.2 mg/ml. Samples of 0.5 ml were withdrawn immediately after mixing and after 5, 15, 30, 60, and 120 min of incubation at 37°. The samples were added to 3 vol of boiling absolute alcohol, boiled 4 min, prepared for assay, and assayed for remaining kinin activity.

Results. Data on 11 incubations of Alcalase with buffered human plasma and EDTA are shown in Table I. Kinin activity was demonstrated in all cases. The maximum activities were found between 5 and 240 min after incubation, varying greatly in the different experiments. Two control incubations of Alcalase with EDTA and phosphate buffer in physiological saline showed no kinin activ-

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TABLE I. Kinin Activity Measured in 4.5 ml of Human Plasma Incubated at 37° for 120 or 240 min with Alcalase (0.2 mg/ml), 0.6 ml of 2×10^{-2} *M* EDTA, and 1.5 ml of 0.5 *M* Phosphate Buffer pH 6.

	Maximum amount of kinins formed	
No. of expt.	Minutes after incubation	Kinin (mμg/ml of plasma)
1	5	79
2	10	65
3	20	291
4	120	444
5	20	116
6	240	672
7	5	62
8	240	417
9	240	1299
10	120	192
11	10	86

ity immediately after mixing or 10, 20, 30, 45, and 120 min after incubation. The activity of the material formed by Alcalase was completely destroyed by chymotrypsin. Trasylol, 2000 units/ml of plasma, decreased the amounts of kinins formed (Fig. 1 and Table II).

The results of incubations of Alcalase with synthetic bradykinin (Fig. 2) demonstrate the kinin-destroying activity of Alcalase. This kinin-destroying activity took place in spite of the mild acid pH..

Discussion. The present data are in accordance with the work of Prado *et al.* (8) who reported that the bacterial protease subtilisin BPN' Nagarse released kinins from heat-treated horse plasma. Recently Butt and Huggins (9) found formation of a vasopressor polypeptide by subtilisin Carlsberg.

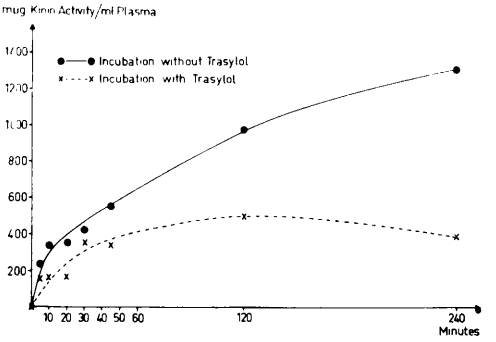

FIG. 1. Kinin-forming activity of Alcalase (0.2 mg/ml); incubation with plasma; EDTA; phosphate buffer, pH 6; with and without Trasylol (2000 units/ml).

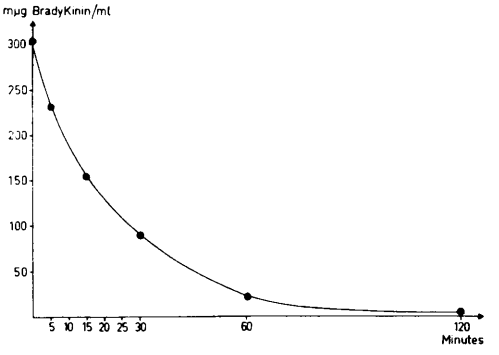

FIG. 2. Kininase activity of Alcalase (0.2 mg/ml); incubation with synthetic bradykinin; EDTA; and phosphate buffer, pH 6.6; in physiological saline. Control incubation of bradykinin, saline, EDTA and phosphate buffer without Alcalase showed 230 mμg of kinin activity/ml after 120 min.

TABLE II. Kinin Activity Measured in 4.5 ml of Human Plasma Incubated at 37° for 240 min with Alcalase, EDTA, and Phosphate Buffer, pH 6, with and without Trasylol (2000 units/ml).

		Kinin activity (mμg/ml of plasma)							
No. of expt.	Trasylol	(min): 0	5	10	20	30	45	120	240
1	+	0	17	23	24	31	28	23	26
	—	0	42	82	145	179	182	192	160
2	+	0	36	41	44	47	25	0	0
	—	0	73	86	31	57	35	—[a]	12
3	+	0	150	158	164	350	337	495	385
	—	0	234	336	349	420	550	976	1299

[a] Less than 19 mμg of kinin activity.

It is, however, not surprising that several vasoactive products may be formed from plasma protein. The complete inactivation by chymotrypsin, found in our study, together with the significant decrease in activity by Trasylol further indicates that kinin formation by Alcalase takes place by activation of the kallikrein system, and suggests that Trasylol may be of value in Alcalase toxicity.

The data of the incubations with synthetic bradykinin show that Alcalase also is a kinin destroying enzyme. This in part explains the highly varying results in our study on kinin formation. It was not possible to prevent kininase activity of Alcalase by adding EDTA and lowering the pH to 6.6. The effect of plasma kininase is abolished by this procedure (10). Varying results in the study on kinin formation may also be due to variations in plasma kininogen. The same plasma, which produced large amounts of kinins in Expt. 3 of Table II also produced large amounts of kinins when treated with Hageman factor activation of kallikrein (10).

Summary. Incubations of human plasma and synthetic bradykinin with the proteolytic enzyme Alcalase demonstrated kinin forming and kinin-destroying properties of this product. Kinin formation by Alcalase was inhibited by Trasylol.

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