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Effects of Chymotrypsin on the Release Reaction and Aggregation of Blood Platelets (40943)

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Abstract. Platelets incubated with chymotrypsin and washed are known to aggregate with fibrinogen. Chymotrypsin is reported to cleave platelet membrane glycoproteins without causing the release reaction seen with some proteolytic enzymes such as trypsin. Recent batches of chymotrypsin from two manufacturers caused platelets to release much of the contents of their dense granules, largely because they are contaminated with trypsin. However, even after inhibition of trypsin with *N*-α-tosyl-L-lysine chloromethylketone hydrochloride (TLCK), chymotrypsin induced some release from platelets at low concentrations of ionized calcium. As expected, platelets incubated with either trypsin-contaminated or TLCK-treated chymotrypsin aggregated with fibrinogen after they were washed. Released or added ADP enhanced the release reaction and development of aggregability with fibrinogen. Thus, [^{14}C]serotonin secretion and fibrinogen-induced aggregation were less when released ADP was enzymatically destroyed during incubation of platelets with 500 μg/ml of trypsin-contaminated chymotrypsin, and they were increased if 25 μ*M* ADP was added before incubating the platelets with 125 μg/ml of this chymotrypsin.

Chymotrypsin-treated platelets bind and aggregate with fibrinogen (1–3). This plasma protein also specifically binds to platelets stimulated with ADP and is essential for ADP-induced aggregation. It seems likely that the same receptor is responsible for both ADP- and chymotrypsin-induced binding (2–6).

Chymotrypsin cleaves platelet membrane glycoproteins (1, 7, 8) but, in contrast to trypsin, Pronase, and papain (9, 10), is reported not to release serotonin and ADP from the dense granules (1, 9–11). Under somewhat different conditions from those used by others, we recently noted that chymotrypsin released both serotonin and ADP from normal human platelets. However, J. F. Mustard, R. L. Kinlough-Rathbone, and M. A. Packham informed us that recent batches of chymotrypsin induced the platelet release reaction under conditions in which earlier batches did not. Since trypsin is frequently present in commercial chymotrypsin preparations (12), we carried out experiments in which *N*-α-tosyl-L-lysine chloromethylketone hydrochloride (TLCK) was added to inhibit the action of trypsin (13). We also assessed the effect of the presence of ADP during incubation with chymotrypsin on the release of [^{14}C]serotonin and subsequent fibrinogen-induced aggregation.

Materials and methods. Unless otherwise noted, the following reagents were dissolved in isotonic saline and stored at −15°: chymotrypsin (Sigma Chemical Co., St. Louis, Mo., and Worthington Biochemical Corp., Freehold, N.J., all about 50 U/mg of protein), 5 mg/ml prepared daily; trypsin (Worthington, type TRL, 100 U/mg), 1 mg/ml prepared daily in 0.05 *M* Hepes-buffered, 0.1 *M* NaCl, pH 7.0; *N*-α-tosyl-L-lysine chloromethylketone hydrochloride (TLCK; Cyclo/Travenol Laboratories, Los Angeles, Calif.), 0.02 *M* prepared each day; creatine phosphate (CP; Calbiochem–Behring Corp., La Jolla, Calif.), 0.1 *M;* creatine kinase (CK; Worthington, 52 U/mg), 2.5 mg/ml prepared daily; apyrase (Sigma, Grade 1), 10 mg/ml; [^{14}C]-serotonin (Amersham Corp., Arlington Heights, Ill., 58 mCi/mmole), 140 μ*M* in 95% ethanol; 0.01 *M* Hepes-buffered modified Tyrode's solution (HBMT), pH 7.4, containing 0.2% bovine serum albumin and no added $CaCl_2$ (11); imipramine-HCl (a gift from Ciba-Geigy, Ardsley, N.Y.), 0.001 *M* dissolved in 0.001 *M* NaOH and stored at 4°; ADP disodium (Sigma), 0.01 *M;* soybean trypsin inhibitor (Worthington), 10 mg/ml

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prepared daily; human fibrinogen (A. B. Kabi, Stockholm, Sweden, grade L), 10 mg/ml in water, dialyzed against isotonic saline buffered to pH 7.5 with 0.01 *M* Hepes.

Preparation of TLCK-treated chymotrypsin and trypsin. Trypsin (1 mg/ml) or chymotrypsin (Worthington CDI 34-D-632) (5 mg/ml) was incubated at room temperature for 60 min with or without 2 m*M* TLCK. No loss of chymotrypsin activity was detected with the specific chromogen 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (14) (Sigma). In contrast, trypsin was inactivated by TLCK since a tenfold dilution failed to release [^{14}C]serotonin from platelets whereas 4 μg/ml without TLCK caused nearly 100% release. Trypsin preparations were diluted to 40 μg/ml in Hepes-saline containing 2 m*M* TLCK before adding 0.1 vol to platelets so that the incubation mixture contained 0.2 m*M* TLCK.

Preparations of platelet suspensions. Gel-filtered human platelets (GFP) were prepared essentially as described elsewhere (11) from blood collected into sodium citrate, acetylsalicyclic acid, and 1/200th part of [^{14}C]serotonin. The platelet count of the suspension was adjusted to 10^9/ml with HBMT. The platelets were disc-shaped, changed to spiny spheres when treated with ADP, and aggregated when shaken in the presence of 0.1 mg/ml fibrinogen and 10 μM ADP.

Release induced by chymotrypsin. Saline or different concentrations of enzyme (0.2 ml) was added to 1.8 ml of the GFP containing 5 μM imipramine, and the mixtures were incubated at 37°. Sometimes 0.5 mg/ml apyrase or 2 m*M* CP plus 6 U/ml CK were present. After 30 min, the samples were placed in chilled glass tubes and centrifuged. The supernatants were used to assess the release of [^{14}C]serotonin (15) or ADP from the platelets. To calculate the former, the percentage of [^{14}C]serotonin in the supernatant of platelets incubated without enzyme was subtracted from the values noted with enzyme. Released ADP was measured by its ability to aggregate platelets in citrated platelet-rich plasma, when aggregation was recorded with an aggregometer (Payton Associates, Inc., Buffalo, N.Y.). The aggregating agent in the supernatants was assumed to be ADP because aggregation was associated with platelet shape change and was prevented by 5 m*M* EDTA and by incubating the supernatant for 5 min with 0.5 mg/ml apyrase. The apyrase had no effect on aggregation when the same final concentration was added directly to the platelet-rich plasma. Release of lactic dehydrogenase was assessed using an LDH-L kit (Bio Dynamics/BMC, Indianapolis, Ind.) by comparing the activity in the supernatant with that found when the whole suspension was frozen and thawed.

Aggregation of enzyme-treated platelets. The incubated suspensions were brought to room temperature. In the initial experiments, soybean trypsin inhibitor was added to samples containing chymotrypsin (2 mg/mg chymotrypsin), but this was found to be unnecessary. The platelets were then washed by adding 10 ml of HBMT brought to pH 6.5 and centrifuging for 20 min at 1500*g*. They were resuspended in 0.2 ml of this HBMT and diluted with 1.2 ml of HBMT, pH 7.4. To study aggregation, 20 μl of 2.5 mg/ml fibrinogen was added to 0.2 ml of each sample, the tubes were shaken at room temperature for about 5 min, and the extent of clumping was assessed macroscopically, 4+ designating a maximal response. Aggregation was sometimes also determined using an aggregometer.

Results. Although [^{14}C]serotonin was not released when GFP were incubated with 125 μg/ml chymotrypsin (Sigma, type IS, lot 108C-8145), 24% was released with 250 μg/ml, and 78% (SEM 2.4) with 500 μg/ml. Without imipramine, all but a trace of the released [^{14}C]serotonin was reincorporated. Between 6 and 10 μM ADP was released with 500 μg/ml chymotrypsin whereas lactic dehydrogenase was not released.

Other lots of recently purchased chymotrypsin (Sigma type II, lot 49C-8015; Worthington type CDI, lot 34D-632) also caused considerable release from human platelets. J. F. Mustard, R. L. Kinlough-Rathbone, and M. S. Packham of McMaster University, Hamilton, Ontario, and the University of Toronto, studied release of [^{14}C]-

TABLE I. EFFECTS OF INCUBATING GEL-FILTERED HUMAN PLATELETS WITH TRYPSIN OR CHYMOTRYPSIN TREATED WITH 2 m*M* TLCK[a]

Additions during incubation			Changes during incubation, [^{14}C]serotonin release (%)	Aggregation with fibrinogen after washing[b]
Enzyme (μg/ml)		TLCK		
None	0	0	0	±
None	0	+	0[c]	0
Trypsin	4	0	92	±
Trypsin	4	+	0	0
Chymotrypsin	500	0	78	2+
Chymotrypsin	500	+	21	2+

[a] After 30 min at 37° with 5 μM imipramine, release of [^{14}C]serotonin was measured. Platelets were then washed and tested for their ability to aggregate with fibrinogen. Average of three experiments.
[b] 4+, maximal, clumping.
[c] The concentration of TLCK incubated with the platelets was 0.2 m*M*. It released 5% [^{14}C]serotonin. This value was used as the blank in samples containing TLCK.

serotonin from washed rabbit platelets (personal communication). In the presence of imipramine and the absence of apyrase, 100 μg/ml of recently purchased chymotrypsin (Sigma type II, lot 109C-8045; Worthington CDI lot 38S-921) released 15 and 77% of platelet [^{14}C]serotonin, respectively, whereas 400 μg/ml of an older lot (Worthington CDI 36S-771) released only 2%.

TLCK treatment abolished the release of [^{14}C]serotonin from human platelets caused by 100 μg/ml trypsin and markedly diminished that caused by 500 μg/ml chymotrypsin (Worthington CDI 34D-632) (Table I). Platelets incubated with this concentration of chymotrypsin with or without TLCK and washed aggregated promptly with fibrinogen (Tables I and II). In contrast, platelets incubated with 4 μg/ml trypsin did not aggregate with fibrinogen. When one of the ADP-destroying enzymes, apyrase or CK with CP, was present during incubation of platelets with 500 μg/ml chymotrypsin, the platelets released only 5 ± 1.6% [^{14}C]serotonin and aggregated less with fibrinogen after they were washed. With 125 μg/ml chymotrypsin, they failed to aggregate with fibrinogen unless 25 μM ADP had been added during incubation with chymotrypsin (Table II).

Discussion. Recent batches of chymotrypsin caused marked release of [^{14}C]-serotonin from human and rabbit platelets whereas earlier batches did not (1, 7–11). Chymotrypsin treated with TLCK, a potent trypsin inhibitor (13), caused con-

TABLE II. EFFECTS OF INCUBATING HUMAN GEL-FILTERED PLATELETS WITH CHYMOTRYPSIN WITH OR WITHOUT ADP-DESTROYING ENZYMES OR 25 μM ADP[a]

Additions during incubation			Changes during incubation		Aggregation with fibrinogen after washing[b]
Chymotrypsin (μg/ml)	Other enzyme[c]	ADP	[^{14}C]Serotonin release (%)	ADP release (μM)	
0	0	0	0	0	0
0	0	+	0		0
125	0	0	0		±
125	+	0	0		0
125	0	+	5		1+
500	0	0	78	8	3+
500	+	0	5	0	1+

[a] After 30 min at 37° with 5 μM imipramine, release of [^{14}C]serotonin and ADP was measured. Platelets were washed and tested for their ability to aggregate with fibrinogen. Typical results of at least three experiments.
[b] 4+, maximal clumping.
[c] Apyrase or CP–CK.

siderably less release, indicating that the trypsin added to activate chymotrypsinogen (12) was partially responsible. As expected, TLCK abolished the effect of 100 μg/ml trypsin on platelets but did not alter the effect of chymotrypsin on a chromogenic titrant.

Chymotrypsin itself is presumably responsible for the release of [^{14}C]serotonin from human platelets caused by 500 μg/ml TLCK-treated chymotrypsin (Table I). This release may not have been observed in earlier studies because our incubation medium had a low concentration of ionized calcium, which facilitates the release reaction of human platelets (16), and because only one group (1) studied serotonin release after adding imipramine. The negative findings of that group may be explained by the presence of apyrase during the incubation, since we noted a reduction of trypsin–chymotrypsin-induced [^{14}C]serotonin release from 78 to 5% in the presence of ADP-destroying enzymes and an enhancement when ADP was added (Table II). A similar potentiation of release by ADP had been noted by others (17). The release of von Willebrand factor from platelet α granules by chymotrypsin (11) may be due to contaminating trypsin.

Like others (1–3), we observed that platelets incubated with chymotrypsin and washed aggregate with fibrinogen. TLCK-treated chymotrypsin was also effective. Studies with ADP-destroying enzymes and addition of ADP indicated that aggregation was most marked when the incubation mixture contained ADP as well as chymotrypsin.

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