PEPTIDASE ACTIVITY OF CARRIER PROTEINS USED IN RADIOIMMUNOASSAYS

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Preparations of lysozyme, bovine serum albumin, and human serum albumin may contain levels of peptidases affecting the stability of angiotensin I and ^{125}I-angiotensin I (Asp^1.Ile^5), the standard and tracer in the radioimmunoassay of angiotensin I. Enzyme activity was evaluated by a radioimmunoassay method measuring the loss of immunoreactivity of angiotensin and an autoradiographic method permitting a quantitation and characterization of fragments of angiotensin. Damage ranged from very slight to extensive and was consistent with chymotrypsin or chymotrypsin-like activity. Evaluation of carrier proteins used in radioimmunoassays is essential for long-term stability of peptides.

In most radioimmunoassays, carrier proteins are employed as diluent proteins to stabilize and protect both standard and tracer and to prevent or minimize the adsorption of highly diluted antigen and antibody to the walls of glass or plastic tubes in which reagents are being stored and assays are being run. Commonly used proteins for this purpose are lysozyme and bovine serum albumin.

The inherent variability of proteins from natural sources, particularly with regard to the presence of peptidases, poses a severe quality-control problem to manufacturers of radioimmunoassay (RIA) kits. Observations of apparent peptidase damage due to carrier proteins have been reported in the radioimmunoassay literature, e.g., for ^{125}I-human chorionic somatomammotropin (*1*), human and porcine ^{125}I-calcitonin (*2,3*), ^{125}I-insulin (*4*), ^{125}I-glucagon (*5*), and ^{125}I-human growth hormone (*6*). Several investigators (*5,7*) have provided rationales for their selection of certain carrier proteins to the exclusion of others. In most reports, however, no supporting evidence is given for the choice of any particular lot or type of carrier protein.

The activity of these contaminating enzymes can have a marked effect upon the stability of reagents and possibly the binding of antigen to antibody. It is, therefore, the purpose of this communication to alert laboratories using RIA to the possibility of the contamination of carrier proteins.

As a result of production difficulties with our plasma renin activity kit (RIA of angiotensin I), we examined a number of commercially available carrier proteins of varying degrees of purification. We have noted that many commercial preparations of lysozyme contain levels of contaminating enzymes affecting the stability of angiotensin I and ^{125}I-angiotensin I (Asp^1.Ile^5), the standard and tracer for the RIA of angiotensin I. Evaluation of samples of BSA and HSA have shown that these are also at times similarly contaminated. In addition, the extent of purification, and consequently cost of these proteins, was not indicative of the absence of contaminating enzymes. Three approaches for the solution of this problem were considered: (A) selection through screening for lots with sufficiently low or no peptidase activity, (B) removal of the peptidases from carrier proteins by specific or nonspecific separation techniques, and (C) inhibition of peptidase activity by addition of appropriate inhibitors, e.g., Trasylol (trademark, Farbenfabriken Bayer) for kallikrein and L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) for chymotrypsin (*8*), and trypsin (*9*).

The third approach appeared to be the most convenient but the use of inhibitors can be costly and may also be a potential source of error in a radioimmunoassay. The first approach was, therefore, investigated for its usefulness in the resolution of the problem.

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Peptidase activities of lysozyme and BSA were evaluated by using ^{125}I-angiotensin I as the substrate and observing the effect of any contaminants on the tracer by radioimmunoassay and by autoradiography. Other radioactive substrates have been described, ^{14}C-acetyl BSA (*10*) to detect peptidase activity at the microgram level and *N*-benzoyl ^{14}C-L-tyrosine ethyl ether (*11*) to detect chymotrypsin at the nanogram level. However, we chose ^{125}I-angiotensin I since this labeled decapeptide was found to be highly susceptible to the action of the peptidases we have observed in carrier proteins.

METHODS

Radioimmunoassay method. Iodine-125-angiotensin I was diluted in 0.1 *M* Tris acetate, pH 7.4, containing 0.1% of the carrier protein. Following incubation at 37°, binding of tracer (50 μl) to antibody was determined by a standard procedure for the RIA of angiotensin (*12*). The carrier protein at this step was generally lysozyme previously found to contain no peptidase activity and the incubation was at 4°. The RIA method measures the loss of immunoreactivity or binding of angiotensin to specific antibodies after incubation at 37°. Since the major fragments of angiotensin I, angiotensin II, and the tetrapeptide, Asp.Arg.Val.Tyr, are known to be nonimmunoreactive (*13*), binding after incubation of tracer with carrier protein constitutes an indirect measure of peptidase activity. Results are summarized in Tables 1 and 2.

Autoradiographic method. The protein carrier (5 mg) in 0.50 ml 0.05 *M* Tris acetate buffer, pH 7.4, containing 1–2 μCi ^{125}I-angiotensin I was incubated for 24 hr at 37°. A higher concentration of both tracer and carrier is required by this method to allow not only quantitation of the individual fragments but also characterization or typing of the enzyme activity from the cleavage pattern (*14*). Aliquots (25–50 μl) of the incubation mixture were applied to Whatman 3MM paper and chromatographed (descending) for 16 hr in the solvent system, 2-butanol-3% NH_3 = 100:44. The spots were visualized by autoradiography (16 hr in contact with x-ray film), excised, and counted in a gamma spectrometer. Results are expressed in Tables 3–7 as percent of the radioactivity in each spot relative to the total radioactivity in all measured spots.

In most cases, three to five radioactive components were present after incubation. The spot marked LP_1 at Rf 0.6 is an unidentified labeled fragment which is less polar than angiotensin I (Rf 0.5). The three major MP (more polar) components consist of angiotensin II (MP_1) at Rf 0.45, an unidentified fragment (MP_2) at Rf 0.3, and the tetrapeptide ^{125}I-Asp.Arg.Val.Tyr (MP_3) at Rf 0.2.

TABLE 1. LYSOZYME—EFFECT OF INCUBATION UPON TRACE BINDING (Bo as %)

Sample	Time at 37° 0	4 hr	1 day	2 days	days
1	—	5.0	—	—	—
2	—	58.6	46.5	—	(8) 12.5
3	—	58.8	51.4	—	21.3
4	—	65.6	43.7	—	8.5
5	—	57.7	39.5	—	6.6
6	65.2	—	61.8	53.0	(7) 28.4
7	63.9	—	38.6	17.0	0.4
8	61.0	—	65.6	63.8	(9) 53.0
9	60.8	—	62.0	56.2	36.0
10	62.7	—	61.9	—	(5) 60.3

TABLE 2. BOVINE SERUM ALBUMIN—EFFECT OF INCUBATION UPON TRACE BINDING (Bo as %)

Sample	Time at 37° 0	4 hr	1 day	2 days	days
1	—	66.6	57.7	—	(8) 60.0
2	—	68.0	57.0	—	48.7
3	69.4	—	68.7	64.7	(9) 53.4
4	72.9	—	70.3	68.0	67.5
5	70.5	—	65.6	57.6	35.4
6	68.4	—	67.3	57.7	40.9
7	64.3	—	52.4	—	(5) 33.6

RESULTS

The peptidase activity in several lots of lysozyme from various sources was determined by the RIA method (Table 1) and by the autoradiographic method (Table 3). The substrate damage ranged from slight in Samples 8 and 10 to extensive in Samples 1, 5, 7, and 11. Similar results were obtained with BSA (Tables 2 and 4) whereas a limited evaluation of three lots of HSA indicated relatively low enzymatic activity (Table 5).

The observed cleavage pattern of ^{125}I-angiotensin I is consistent with chymotrypsin or chymotrypsin-like enzyme activity in the lysozyme and BSA which we examined. The dose-related inhibition of the high enzymatic activity of lysozyme Sample 11 with TPCK supports this conclusion (Table 6). The addition of α-, γ-, or ∂-chymotrypsin at the nanogram level approximated qualitatively the fragmentation pattern of the observed peptidase activity in contaminated lysozyme. Trypsin and carboxypeptidase caused no significant dose-dependent degradation while β-chymotrypsin was highly destructive at the

TABLE 3. PEPTIDASE ACTIVITY IN LYSOZYME

Sample	Percent composition of incubation mixture				
	LP_1	A-I	A-II	MP_2	MP_3
1	2	30	55	4	9
2	6	65	22	—	7
3	4	55	27	9	5
7	2	52	31	3	12
8	7	63	19	8	2
11	—	1	7	—	92
Control*	2	89	6	2	1
Control†	1	92	5	1	1

* Substrate incubated in buffer only.
† Nonincubated (frozen) buffered substrate.

TABLE 4. PEPTIDASE ACTIVITY IN BOVINE SERUM ALBUMIN

Sample	Percent composition of incubation mixture				
	LP_1	A-I	A-II	MP_2	MP_3
2	3	38	9	35	15
3	3	82	6	5	4
4	10	73	17	—	—
6	12	32	17	29	10
Control*	1	89	6	2	1
Control†	1	92	5	1	1

* Substrate incubated in buffer only.
† Nonincubated (frozen) buffered substrate.

TABLE 5. PEPTIDASE ACTIVITY IN HUMAN SERUM ALBUMIN

Sample	Percent composition of incubation mixture				
	LP_1	A-I	A-II	MP_2	MP_3
1	4	76	10	4	6
2	10	44	13	29	4
3	2	78	3	8	9
Control*	1	90	5	1	3
Control†	1	92	5	1	1

* Substrate incubated in buffer only.
† Nonincubated (frozen) buffered substrate.

TABLE 6. EFFECT OF TPCK ON PEPTIDASE ACTIVITY IN LYSOZYME SAMPLE 11

mg TPCK	Percent composition of incubation mixture*				
	LP_1	A-I	A-II	MP_2	MP_3
0	—	1	7	—	92
0.017†	—	7	32	—	61
0.17‡	—	42	27	—	32

* Incubated 3.5 hr at 37°.
† Lysozyme in 0.05 M phosphate, pH 7.4, preincubated for 10 min with 0.017 mg TPCK in 1 μl acetone.
‡ Lysozyme preincubated with 0.17 mg TPCK in 10 μl acetone.

TABLE 7. EFFECT OF TRASYLOL ON PEPTIDASE ACTIVITY IN LYSOZYME

Sample	KIU Trasylol	Percent composition of incubation mixture*				
		LP_1	A-I	A-II	MP_2	MP_3
8	0	4	82	9	4	1
	1	4	83	9	2	2
	10	4	83	9	2	2
	100	4	84	8	2	2
	1,000	4	84	9	2	1
11	0	1	3	19	3	74
	1	2	4	44	5	45
	10	2	4	46	5	43
	100	2	12	52	7	27
	1,000	5	56	30	5	4
Control†		5	80	10	2	4
Control‡		5	86	7	1	1

* Incubated for 3 days at 37°.
† Substrate incubated in buffer only.
‡ Nonincubated (frozen) buffered substrate.

0.5-ng level. Trasylol, 1,000 KIU, was an effective inhibitor of α, γ, and ∂-chymotrypsin as well as of the highly destructive enzymes in lysozyme Sample 11 (Table 7). The appearance of the unidentified MP_2 component in some BSA and HSA samples suggests greater diversity of enzyme contaminants (Tables 4 and 5).

DISCUSSION

The susceptibility of peptide antigens and hormones to peptidases varies and depends on the availability and the accessibility of vulnerable peptide bonds relative to the more plentiful but possibly less accessible sites in the carrier protein.

It is appropriate to differentiate between the action of the peptidases in carriers which cause "carrier" damage and action of proteolytic enzymes, normally present in plasma or serum, which cause "incubation" damage. Carrier damage may occur in the labeled and the unlabeled antigens during storage at room temperature for brief periods of time and to a lesser extent at refrigerator temperature. In one instance of severe contamination, however, cooling to —20° did not prevent degradation of angiotensin during storage for 20 days. Hence, carrier damage adds to incubation damage and may not be inhibited by addition of specific or nonspecific inhibitors to plasma or serum before incubation.

The observed rapid destruction of angiotensin I in the presence of a considerably larger amount of lysozyme indicates a high degree of substrate sensitivity. Similar sensitivities probably apply to other peptide hormones which have been reported to undergo loss of immunoreactivity due to peptidase damage. In the case of ^{125}I-angiotensin I, the ob-

served cleavage pattern suggests chymotrypsin or chymotrypsin-like activity as the primary cause of "carrier" damage. Enzymes such as trypsin and carboxypeptidase were either absent or considerably less discriminating.

Instead of exerting a stabilizing and protective effect, the use of contaminated carriers leads to an accelerated deterioration of labeled and unlabeled peptides. To complicate matters, the investigation has shown no general correlation between apparent quality of the carrier in terms of crystallinity, extent of purification, and even cost and the absence of peptidases. A partially purified lot of protein may show less peptidase activity than a more expensive, highly purified material from the same supplier. There is also some evidence of intrabatch, bottle-to-bottle variability. These findings suggest possible cross-contamination of solutions during processing and, to a lesser extent, during packaging of the final product. The need to evaluate every lot or preferably every container of carrier protein either by ^{125}I-angiotensin I or other suitable substrates is essential for long-term stability of peptides.

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