TrypLE Select Enzyme: a temperature-stable replacement for animal trypsin in cell dissociation applications

Abstract

Gibco™ TrypLE™ Select Enzyme is a recombinant fungal trypsin-like protease that has proven effective at dissociating many different attachment-dependent mammalian cell lines. It has dissociation kinetics similar to those of porcine trypsin and exhibits lower cell toxicity. Cell replating, proliferation kinetics, and long-term maintenance were comparable to cells harvested using animal trypsin. HPLC analysis indicates a single peak corresponding to the active form of the recombinant enzyme, as opposed to multiple peaks seen with animal (porcine pancreatic) trypsin.

Studies of TrypLE Select Enzyme in solution have demonstrated remarkable enzyme stability. Enzyme assay results indicate that at 4°C and room temperature, the enzyme remains stable for more than 12 months. Even at 37°C, TrypLE Select Enzyme maintains 85% activity for 8 days. In contrast, porcine trypsin loses >95% of its trypsin activity after just 24 hr at 37°C. This temperature stability will allow more convenient storage and handling of this cell dissociation enzyme during routine cell culture procedures.

Introduction

TrypLE Select Enzyme is a high-purity, recombinant fungal enzyme produced by fermentation. It is known to be a serine protease with trypsin-like activity (i.e., it cleaves at the same two amino acid sites—arginine and lysine—as trypsin, and has a pH activity profile similar to that of trypsin). This activity suggests that TrypLE Select Enzyme will substitute optimally for traditional porcine trypsin to dissociate cultured cells. TrypLE Select Enzyme is available as 1X and 10X solutions prepared in phosphate-buffered saline (PBS) with 1 mM EDTA. The 10X solution can be diluted to 1X using the same buffers. All studies shown here were conducted with 1X TrypLE Select Enzyme.

Porcine trypsin is unstable when stored unfrozen and suffers from relative instability at and above room temperature. Data suggest that TrypLE Select Enzyme has superior stability compared to porcine trypsin.

This study was designed to determine the purity and storage stability of TrypLE Select Enzyme; the concentration at which it should be used; whether the enzyme needs to be inactivated with an inhibitor; if it is toxic to cells in the short or long term; and its ability to work on multiple cell lines.

Materials and methods

Ac-Arg-*p*NA protocol for determining tryptic activity

The acetyl arginine *p*-nitroaniline (Ac-Arg-*p*NA) enzyme assay was used to determine the activity of microbial trypsin-like enzymes [1]. The liberated *p*NA produces an absorption increase at 405 nm that is proportional to the enzyme activity. The TrypLE Select Enzyme samples were formulated at a 1X working concentration in PBS with 1 mM EDTA. The results are given in rPU/mL.

Recombinant protease activity unit (rPU)

One rPU is the quantity of enzyme that will convert 1.0 mmol of Ac-Arg-*p*NA substrate per minute at pH 8.0 and room temperature (22 ± 1°C). Conversion factor: 1 rPU is approximately 293 USP trypsin units.

HPLC protocol

The chromatographs shown in Figure 5 (See "Results") illustrate typical Hamilton PRP-3 column separations of trypsin samples. Mobile phase A (0.1% trifluoroacetic acid in HPLC-grade water) and mobile phase B (0.1% trifluoroacetic acid in HPLC-grade acetonitrile) were used to wash and prepare the column for separation. A known trypsin standard was used to create a standard curve (correlation coefficient R^2 >0.999).

Cell line

The following cell lines were tested using TrypLE Select Enzyme (both serum-free and serum-supplemented media were used): Madin-Darby canine kidney (MDCK), human lung carcinoma (A549), African green monkey kidney (VERO), porcine kidney (PK-15), Madin-Darby bovine kidney (MDBK), human embryonic kidney (293F MSR), and Chinese hamster ovary (CHO-K1). These cell lines were tested for multiple-passage toxicity and serial dilution dose–response curves.

Serial dilution protocol

The optimal concentration of the final product was determined by serial dilution experiments. A series of ten 2-fold dilutions of TrypLE Select Enzyme were tested on cells to determine the half maximal concentration. Cells were treated with serially diluted enzyme for a fixed time, and dissociated cells were counted to determine total viable cells/mL. The half maximal (or 50% activity) concentration was determined from the dose–response curve.

Short-term toxicity protocol

MDCK cells were treated with TrypLE Select Enzyme and left for 270 min. Cell viability was obtained at 30 and 270 min to test for possible toxicity. (Normal exposure time for MDCK cells is 30 min.)

Multiple-passage toxicity protocol

Cells were plated in T-25 flasks at appropriate densities and in appropriate media for the cell types. After the cells were washed with DPBS, 1 mL of TrypLE Select Enzyme or porcine trypsin control was added to each T-25 flask. After all cells dissociated from the plastic, the cells were washed with growth medium and centrifuged at 1,000 rpm for 5 minutes. The cell pellet was resuspended in growth medium, and total viable cell counts were determined. The cells were seeded into new flasks, and the assay was repeated for six consecutive passages. The flasks were incubated at 37°C with 5–8% CO_2 (depending on cell line) and subcultured over 3- and 4-day passages. Consecutive day 3 and day 4 passages initiated using a constant seed density were averaged, and an acceptance criterion of ≥80% of control was used to assess performance of TrypLE Select Enzyme. Test samples were stored in the dark while not in use.

Plating efficiency protocol

MDCK and A549 cells were used to test for colony plating efficiency. Cells were grown in appropriate growth medium and plated in 6-well plates at 100 and 200 cells/well. Colonies were stained with a 0.5% methylene blue solution and counted after 7 or 11 days (depending on cell line). Results were based on colony counts compared to control wells.

Trypsin inhibitor

MDCK cells were treated with traditional animal trypsin or TrypLE Select Enzyme and replated into new T-25 flasks in Gibco™ OptiPRO™ Serum-Free Medium (SFM). Cells were not washed following dissociation, and no protease inhibitors were used.

Results

Ac-Arg-*p*NA assay

The *p*NA enzyme was used to determine the activity of microbial trypsin-like enzymes for the following:

- **Freeze/thaw stability:** No loss in activity was seen on 4 different lots following 6 freeze/thaw cycles (Figure 1).
- **12-month shelf-life:** Enzyme activity did not drop after 12 months of storage at –20°C, 4°C, or ambient temperatures (all samples stored in the dark) (Figure 2).
- **Stability comparison:** TrypLE Select Enzyme showed superior stability compared to purified porcine trypsin stored at 37°C (Figures 3 and 4).

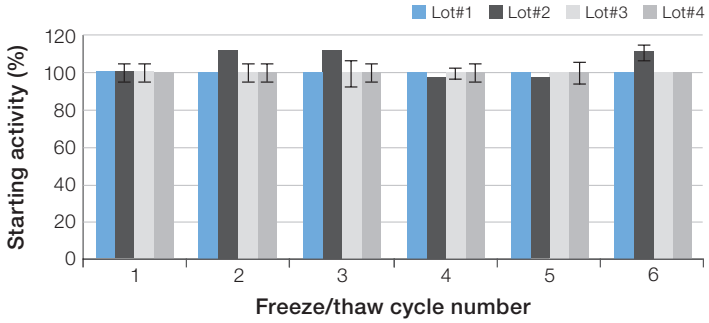

Figure 1. Freeze/thaw stability. No loss in enzyme activity was seen on 4 different lots of TrypLE Select Enzyme following 6 freeze/thaw cycles.

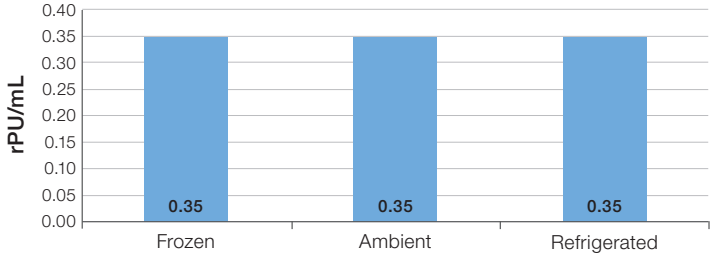

Figure 2. Shelf-life data over 12 months. TrypLE Select Enzyme activity did not drop after 12 months of storage at frozen, ambient, or refrigerated storage temperatures (in the dark).

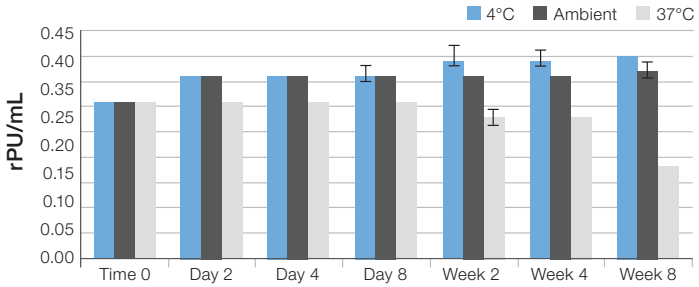

Figure 3. TrypLE Select Enzyme stability. TrypLE Select Enzyme shows superior stability compared to crude or purified porcine trypsin at various storage temperatures. Even after 8 weeks at 37°C, 50% enzyme activity was still seen. All samples were stored in the dark.

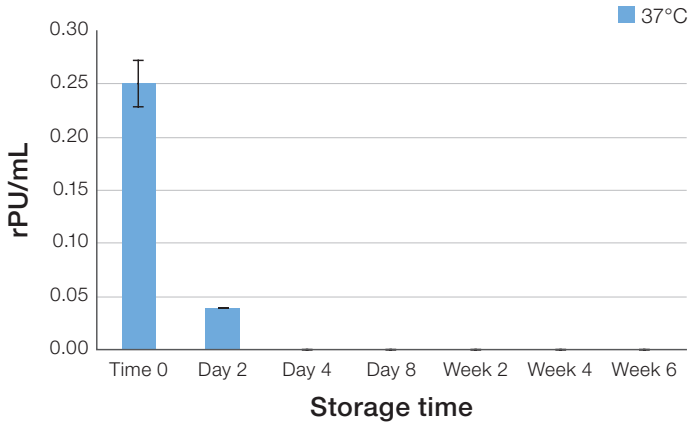

Figure 4. Purified porcine trypsin stability. Enzyme activity decreased rapidly in samples of purified porcine trypsin stored at 37°C.

Enzyme purity

As seen in the Figure 5 chromatograph, the multiple peaks for porcine trypsin indicate impurities. The TrypLE Select Enzyme chromatograph shows a single peak demonstrating the purity of TrypLE Select Enzyme.

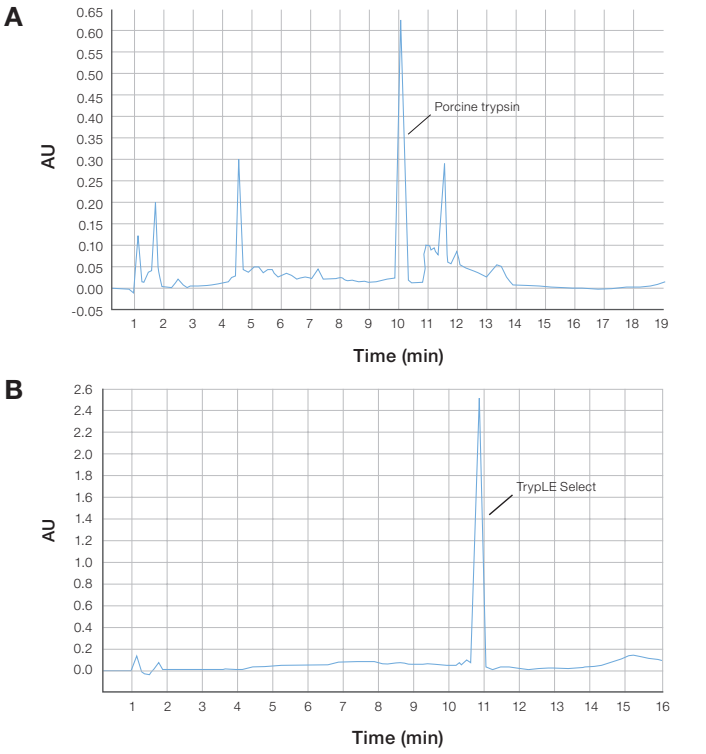

Figure 5. Enzyme purity. As seen in the HPLC chromatographs, **(A)** multiple peaks for porcine trypsin indicate impurities, and **(B)** a single peak for TrypLE Select Enzyme demonstrate its superior purity.

Serial dilution protocol

The dose–response curve shown in Figure 6 is a representative of all the cell lines tested. The half maximal concentrations for both trials shown in the example were between 0.01X and 0.025X, well below the selected final working concentration of 1X. The 1X concentration was used in all experiments described. Similar results were found for all cell lines tested.

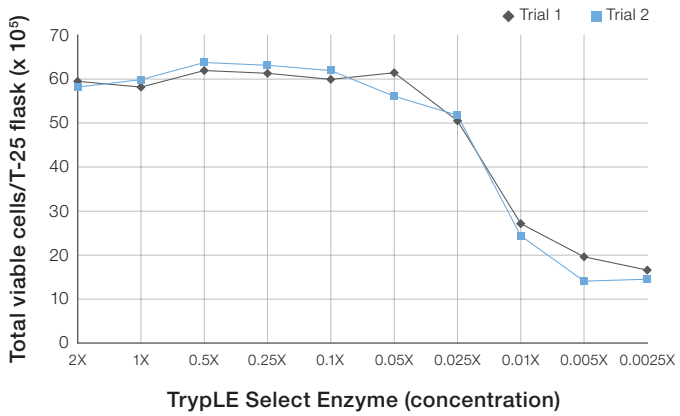

Figure 6. Example of dose–response curve. The half maximal concentrations for both trials shown were between 0.01X and 0.025X, well below the selected final working concentration of 1X.

Cell removal by enzymes stored at 37°C

As seen in Figure 7, the purified porcine trypsin failed to remove cells after 3 days, mirroring the rapid loss in enzyme activity as seen in Figure 4. Even after 50% loss of enzyme activity (Figure 3), the week 8 TrypLE Select Enzyme sample removed cells just as efficiently as the time 0 sample.

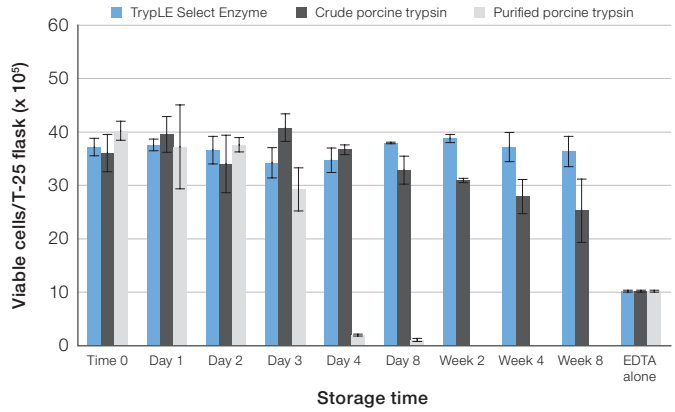

Figure 7. Effect of 37°C storage on cell removal by TrypLE Select Enzyme, crude porcine trypsin, and purified porcine trypsin. The purified porcine trypsin failed to remove cells after 3 days, mirroring the rapid loss in enzyme activity as seen in Figure 4. Crude porcine trypsin starts to lose activity almost immediately but still removes cells after 8 weeks of incubation, possibly due to the action of the EDTA and contaminating proteases such as chymotrypsin and collagenase. Even after 50% loss of enzyme activity (Figure 3), the week 8 TrypLE Select Enzyme sample removed cells just as efficiently as the time 0 sample.

Short-term toxicity

No loss in MDCK cell viability was observed after 270 min of exposure to TrypLE Select Enzyme. Normal exposure time for trypsin in MDCK cells is 30 min (Figure 8).

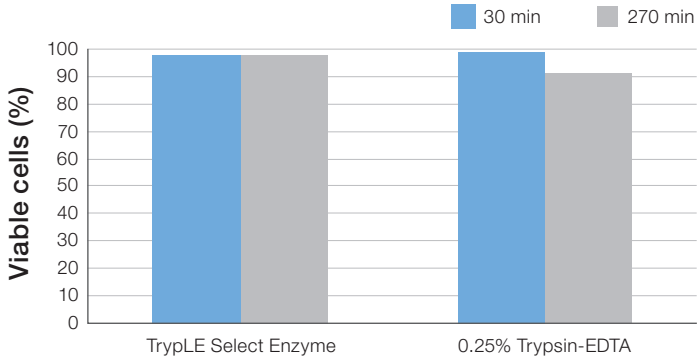

Figure 8. Short-term toxicity study on MDCK cells. No loss in cell viability was observed after 270 min of exposure to TrypLE Select Enzyme. Normal exposure time for MDCK cells is 30 min.

Multiple-passage assays

As seen in Table 1, all cell lines tested met the acceptance criterion of ≥80% of control. Cell viability remained >95% in all cell lines tested. Test samples were stored in the dark while not in use.

Table 1. Efficiency of TrypLE Select Enzyme in multiple-passage assays on many common cell lines.

Cell line tested	Gibco™ growth medium	Mean time required for cell release (min)	Mean viability (%)	Mean cell yield expressed as percent of porcine trypsin control (%)
VERO	VP-SFM	5	100	104
VERO	EMEM + 5% FBS	8	99.9	98
VERO	OptiPRO SFM	7	98	130
PK-15	EMEM + 5% FBS	27	98	101
PK-15	OptiPRO SFM	11	98.8	106
MDCK	OptiPRO SFM	28.5	98	87
MDBK	DMEM + 5% FBS	15	100	94
A549	DMEM + 5% FBS	9	98	114
293F	DMEM + 5% FBS	2	97.3	131
CHO-K1	CHO Cell Culture Medium	7	95.3	96

Colony plating efficiency

For both A549 and MDCK cell lines, plating efficiency was >100% of control (Figure 9).

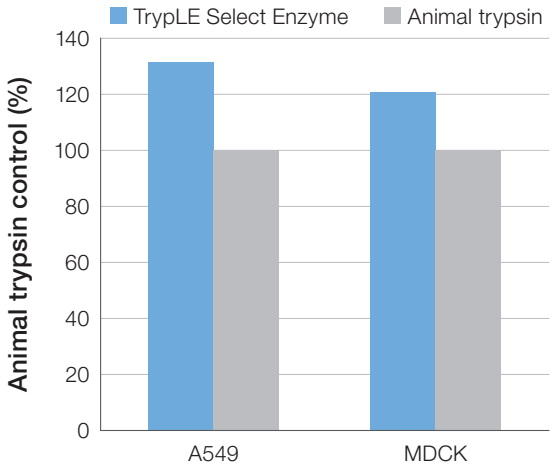

Figure 9. Colony plating efficiency. For both cell lines (A549 and MDCK) tested, plating efficiency was >100% of the porcine trypsin control.

No need for trypsin inhibitor

MDCK cells treated with traditional animal trypsin did not attach or spread out after 24 hours. Cells treated with TrypLE Select Enzyme formed colonies and had good morphology after 24 hr (Figure 10). However, if viability decline is observed with some cell lines, defined trypsin inhibitor (Cat. No. R007100) may be used to inactivate TrypLE Select Enzyme.

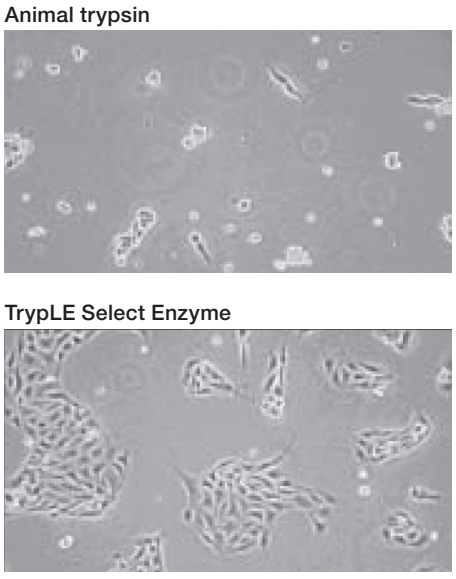

Figure 10. MDCK cell dissociation without washing or use of trypsin inhibitors. Cells were not washed following dissociation, and no protease inhibitors were used. Morphology was observed 24 hr later. While 1 mM EDTA was needed for TrypLE Select Enzyme to remove cells, protease inhibitors were never needed to preserve cell viability or plating efficiency. In fact, use of inhibitors reduced viability and growth rate at some concentrations.

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Discussion

Results from the various assays presented here indicate that TrypLE Select Enzyme performs equivalent to or better than animal trypsin, and is therefore a suitable, nontoxic, stable replacement for cell removal applications. The in-house shelf-life testing program at Thermo Fisher Scientific has demonstrated that TrypLE Select Enzyme can be stored at 4°C or ambient temperature (in the dark) for at least 12 months with no loss in activity or cell removal kinetics. Incubation data (37°C) indicate that TrypLE Select Enzyme is extremely stable compared to porcine trypsin, which makes it much more convenient for routine cell culture use.

TrypLE Select Enzyme exhibits only one protein peak by HPLC, which represents the trypsin-like enzyme. The purified porcine trypsin activity and protein peak disappear after 1 week at 37°C, while TrypLE Select Enzyme still has 100% enzyme activity. Only 50% of the enzyme activity is lost after 8 weeks at 37°C.

TrypLE Select Enzyme is stable when subjected to repeated freeze/thaw cycles. It has the same amino acid cleavage site as porcine trypsin and a similar pH activity profile (see patent information [2,3]). TrypLE Select Enzyme maintains cell viability and plating efficiency, and it has been tested on multiple cell lines with results comparable to porcine trypsin.

In summary, TrypLE Select Enzyme is superior to animal trypsin in purity, stability, and plating efficiency. Cell removal and proliferation kinetics are also comparable to animal trypsin.

References

1. Internal QC Test: Determining Activity for Microbial Trypsin-like Protease Using Ac-Arg-*p*NA.
2. Patent 5,288,627: Endoprotease from Fusarium oxysporum DSM 2672 for Use in Detergents.
3. Patent 5,693,520: Recombinant Trypsin-Like Protease.

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